IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of	**MAIL STOP RCE**
Pierre Jurdic et al.	Group Art Unit: 1633
Application No.: 10/559,819	Examiner: Quang Nguyen
Filed: June 2, 2006	Confirmation No.: 9643
For: PATTERNS FOR A SKELETAL SYSTEM	

DECLARATION OF PIERRE JURDIC UNDER 37 CFR 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I submit this Declaration in support of the referenced patent application:

1. I am the named inventor in the referenced application. My curriculum vitae is attached hereto as Attachment A.

2. I have read the Final Official Action of January 4, 2010, and the Advisory Action of May 11, 2010.

3. Osteoclasts derive from precursor cells found in hematopoietic tissue. The transition between precursor to the mature state of such cells occurs through a process known in the art as "differentiation." In the case of the osteoclast, differentiation is effected under the action of two growth factors, i.e., M-CSF and RANK-L. It is characterized by a transition of a mononuclear cell to a functional osteoclast. The functional osteoclast is generally known in the art as a mature or *differentiated* osteoclast. It is multinuclear, and expresses a number of biochemical markers. It is well known in the art that only differentiated osteoclasts are able to resorb bone tissue.

4. Those principles, and art-accepted usage of that terminology is illustrated in the literature. For example, Teitelbaum et al., describe the development and differentiation of osteoclasts, and their role in bone resorption:

> Bone remodeling is a continuous process that is responsible for normal bone turnover. The process is initiated by the recruitment of HEMATOPOIETIC osteoclast precursor to bone, where they mature into resorptive POLYKARYONS. The differentiated osteoclasts generate a resorption lacunae ('pit') that is ~50 μm deep, then are replaced by osteoblasts that deposit new bone in the cavity. The boundaries of the resorption lacunae are permanently marked by a METCHROMATIC CEMENT LINE. When the skeleton is in balance, the amount of new bone deposited is equal to that previously resorbed. In states of net bone loss, such as osteoporosis, more bone is resorbed than is subsequently deposited.

Teitelbaum, S.L., et al., Genetic Regulation of Osteoclast Development and Function, Nature Reviews/Genetics, 4, 638-649, at 640, Box 1 (Aug. 2003). *See also*, GuezGuez, A, et al., 3BP2 Adapter Protein is required for RANKL-Induced Osteoclast Differentiation of RAW264.7 Cells, J. Biol. Chem., http://www.jbc.org/cgi/doi/10.1074/jbc.M109.091124 (May 3, 2010) ("Osteoclasts are multinucleated bone-resorbing cells, differentiating from Cd11b+ hematopoietic cells of the monocyte/macrophage lineage.").

5. The art generally, and particularly the foregoing references, demonstrate that the term "differentiated osteoclasts" is a common and accepted term within the art, and that one skilled in the art would readily understand its meaning. The referenced application, and the claims, use the term in a manner consistent with the usage in the art generally, and consistent with the usage of the foregoing references.

6. With regard to the cited prior art, the Van Blitterswijck reference is distinct from the claimed invention for a number of reasons. First, it describes the use of undifferentiated cells, and placing those cells on a matrix. It fails to disclose a co-culture of osteoblasts and osteoclasts; and it fails to disclose the use of osteoblasts at confluence on the support. One skilled in the art reading Van Blitterswijck would have understood that those cells are not differentiated to osteoblasts and osteoclasts, and thus are not the same cells. One skilled in the art would not find within the Van Blitterswijck reference any report of or recommendation to differentiate those cells into osteoblasts and/or osteoclasts, less so the combination as required by the claims, and still less at the claimed concentration.

7. It is well known in the art that differentiation of precursor cells to osteoclasts requires the presence of RANK-L. The Van Blitterswijck reference does not reference or suggest the use of RANK-L. One skilled in the art would have reasonably concluded that the authors did not use RANK-L, and thus the skilled artisan would have concluded that the disclosed method could not have effected *in vitro* formation of osteoclasts.

8. On information and belief, the claims are rejected in reliance on Shibutani et al., J. Biomed. Mater. Res., 50:153-159 (2000) in combination with Chambers et al., J. Cell. Sci., 76, 155-165 (1985). I am familiar with both the cited Shibutani and Chambers references. Among other things, the Chambers reference speculates that osteoblasts are able digest osteoid *in vitro*. Chambers acknowledges, however, that "there was no evidence that such cells were capable of resorption of bone mineral...." Chambers at p. 165. The art has demonstrated that such speculation is not merely unfounded, but incorrect. *E.g.,* Teitelbaum, S.L., Osteoclasts: Culprits in Inflammatory Osteolysis, Arthritis Res. Ther., 8(1), 201 (2006), Epub 2005 Nov. 29, Abstract ("The osteoclast, which is a member of the

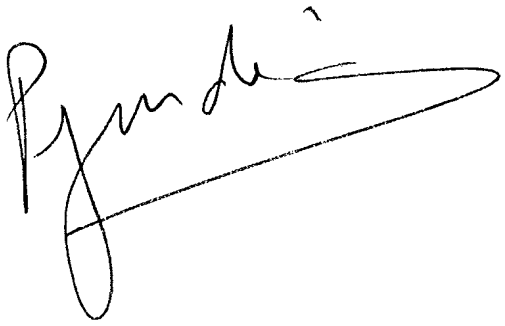
monocyte/macrophage family, is the exclusive bone resorptive cell, and its differentiation and activation are under the aegis of a variety of cytokines."); *see also* Karsenty, G., The Genetic Transformation of Bone Biology, Genes & Development, 13:3037-3051, at 3037 (1999), Cold Spring Harbor Laboratory Press; and Komori, T., Regulation of Osteoblast Differentiation by Transcription Factors," J. Cell. Biochem., 99:1233-1239 (2006). Those references are consistent in refuting the speculative statement of Chambers, and their findings are generally accepted in the art as scientifically valid.

9. In view of Chamber's clearly unsupported speculation, and particularly in view of the subsequent refutation of that speculation, one skilled in the art would not have had a well reasoned basis for modifying the Shibutani model by adding osteoblasts; and less so for actually seeding osteoclasts on a layer of confluent osteoblasts and/or osteoblast nodules on a mineralized matrix.

10. I am also familiar with Rovira et al., Biomaterials, 17, 1535-1540 (1995). Rovira describes a biomaterial and asserts its utility as a substitute for bone tissue in orthopedic implants. Rovira addresses colonization of the material by osteoblasts. However, Rovira does not address the relationship of that material and osteoblasts with osteoclasts; nor does it address the functional relationship of osteoblasts and osteoclasts on such material; nor does it address whether osteoclasts, seeded on a confluent layer of osteoblasts, would be able to penetrate and make their way through that layer; nor does it address the suitability of the purportedly new biomaterial for use in an *in vivo* system for diagnosing or assaying therapies for bone-related diseases.

11. I declare that all statements made herein of my own knowledge are true; and that all statements made on information and belief I believe to be true. Further, I understand that any willful false statements and the like made herein are punishable by fine or imprisonment, or both (18 U.S.C. 1001), and that any such statements may jeopardize the validity of the application or any patent issuing thereon.

Date: July 12, 2010 By: Pierre Jurdic



CURRICULUM VITAE

Family name : JURDIC

First name : Pierre

Date and place of birth : March 7th, 1951

Ste Colombe/Vienne , Rhône , FRANCE

Nationality : French

Position : Directeur de recherche INSERM; Group leader

Lab address : Ecole Normale Supérieure de Lyon

Institut de Genomique Fonctionnelle de Lyon

UMR 5242 CNRS; 46, Allée d'Italie

69364 LYON Cédex 07 - FRANCE

Telephone : 04.72.72.81.68; **Fax**: 04-72-72-80-80; **e-mail**: pjurdic@ens-lyon.fr

Previous positions out of France :

2/1981 - 6/1984 : Postdoctoral Fellow in Dr C. Moscovici's Laboratory, University of Florida, USA

3/1987 - 7/1987: Exchange visitor in Pr. Toyoshima's lab (The Institute of Medical Science, University of Tokyo, Japan),)

8/1990 - 8/1991: Sabbatical year in Pr. E. Lazarides's laboratory at the California Institute of Technology (Caltech,; Pasadena, CA, USA).

Previous positions :

1/1983 - 7/1984: Chargé de recherche INSERM dans le laboratoire du Pr C. Moscovici (USA).

7/1984-12/1987: Chargé de recherche INSERM dans le laboratoire de J. Samarut; Département de Biologie Générale et Appliquée (Professeur J. Godet); Université Lyon 1

1/1988-12/1992: Chargé de recherche INSERM dans le laboratoire de J. Samarut; Département de Biologie Moléculaire et Cellulaire; Ecole Normale Supérieure de Lyon

1/1993- présent : Directeur de recherche INSERM; Responsable de l'équipe »Cell biology and bone physiopathology » in Institut de Génomique Fonctionnelle de Lyon ; Ecole Normale Supérieure de Lyon

1/2009-present : Directeur of the Institut de Recherche Fédératif (IFR) 128 Biosciences Gerland-Lyon Sud

Scientific societies

P. Jurdic. January 2001

- President of the French Society for Cell Biology; Cofounder of "Journées Françaises de biologie des tissus minéralisés"
- Member of the American society for cell biology and American society for bone and mineral research; European calcified Tissue Society

Scientific expertise

Editorial board : Biology of the Cell

Occasional reviewer for J. Cell Science ; J. Bone and Mineral Res., ;Experimental Cell Research ; Bone ; Mol. Biol. Cell ; J. Cell Biol., Oncogene, Bone; Mol. Biol. Cell, Stem Cell, Odontology, Int. J. Dev. Biol., Pharmacological journal, Trends Cell Biol., J. Cell Biology, J. Clin. Invest., PLoSOne

Member of the study section of ARC (French agency against cancer)

Member of the Avenir committee of INSERM

P. Jurdic. January 2001

GENETIC REGULATION OF OSTEOCLAST DEVELOPMENT AND FUNCTION

Steven L. Teitelbaum and F. Patrick Ross

Osteoclasts are the principal, if not exclusive, bone-resorbing cells, and their activity has a profound impact on skeletal health. So, disorders of skeletal insufficiency, such as osteoporosis, typically represent enhanced osteoclastic bone resorption relative to bone formation. Prevention of pathological bone loss therefore depends on an appreciation of the mechanisms by which osteoclasts differentiate from their precursors and degrade the skeleton. The past five years have witnessed important insights into osteoclast formation and function. Many of these discoveries have been made through genetic experiments that involved the rare hereditary disorder osteopetrosis.

OSTEOCLASTOGENESIS
The generation of osteoclasts from their monocytic precursors.

POLARIZATION
The capacity of the osteoclast to generate asymmetric protein distributions and shapes, which allow it to attach to and resorb bone.

VACUOLAR PROTON PUMPS
Ruffled-membrane-residing electrogenic H^+ATPases that transport protons into the resorptive microenvironment.

Department of Pathology, Washington University School of Medicine, 660 South Euclid Avenue, Campus Box 8118, St. Louis, Missouri 63110, USA.
e-mails: teitelbs@medicine.wustl.edu; rossf@medicine.wustl.edu
doi:10.1038/nrg1122

Despite the common misconception that the skeleton is immutable, it is, in fact, an ever-changing organ in which mass and shape are dictated by the activities of osteoblasts (the cells that are responsible for bone formation) and osteoclasts (the cells that are responsible for bone resorption). So, any net loss of bone tissue reflects the extent to which osteoclastic bone resorption exceeds the bone-forming capacity of osteoblasts.

To develop pharmaceutical agents that arrest the progression of skeletal disorders such as osteoporosis, we need to know how osteoclasts are generated (OSTEOCLASTOGENESIS) and how they degrade bone. In the past five years, remarkable progress has been made in dissecting the molecular mechanisms of osteoclastogenesis and bone degradation, which largely reflects the results of genetic studies of experimental animals or patients with abnormal bone phenotypes. These genetic observations have led to the discovery of new therapeutic targets for the prevention and treatment of osteoporosis, which raises the real possibility of a cure for this disease. Here, we review the genetic regulation of the osteoclast — a cell that is central to the pathogenesis of osteoporosis.

Given the crucial role of the study of abnormal bone phenotypes in the dissection of the genetic regulation of osteoclast development and function, we have organized this review according to the different categories of phenotypes. We start by discussing osteoclasts and their relationship to bone diseases in general, before moving on to the specific classes of osteoclast-related disorders: arrest of osteoclastogenesis, arrest of osteoclast function and osteoclast gain of function. We then discuss the influence of the immune system on the osteoclast, before summarizing our present knowledge of the genetic control of the cell.

Osteoclasts and bone disorders

The osteoclast is a member of the monocyte/macrophage family, which is distinguished by its POLARIZATION (induced by contact with bone) and its expression of specific proteins such as cathepsin K (FIG. 1). The most notable feature of the polarized osteoclast is its ruffled membrane. This structure, which is the resorptive organelle of the cell, consists of a unique villous-like complex of the plasma membrane that is juxtaposed to bone and contains numerous 'spike-like' VACUOLAR PROTON PUMPS (H^+ATPases). Formation of the osteoclast ruffled membrane is dependent on contact with bone and is only apparent when the cell degrades the skeletal matrix[1] (BOX 1; FIG. 1). Too little osteoclast activity, which causes increased bone mass (osteopetrosis), or too much osteoclast activity, which causes decreased bone mass (osteoporosis), both lead to bone disorders.

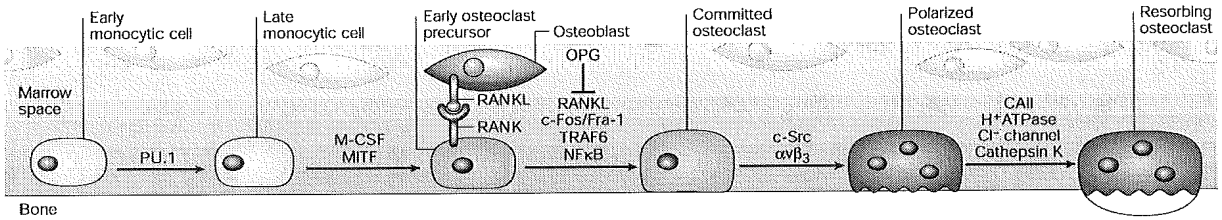


Figure 1 | **Regulation of osteoclast formation and function.** The osteoclast is a member of the monocyte/macrophage family. Early nonspecific differentiation along the osteoclast pathway is dependent on PU.1 and the MITF family of transcription factors, as well as the macrophage proliferation and survival cytokine M-CSF. Activation of RANK by osteoblast-expressed RANK ligand (RANKL) commits the cell to the osteoclast fate, which is mediated by signalling molecules such as AP-1 transcription factors, tumour necrosis factor receptor associated factor 6 (TRAF6), nuclear factor κB (NFκB), c-Fos and Fra-1. RANKL-stimulated osteoclastogenesis is inhibited by the RANKL decoy receptor osteoprotegerin (OPG). The initial event in development of the resorptive capacity of the mature osteoclast is its polarization, which requires c-Src and the αvβ3 integrin. Once polarized, the osteoclast mobilizes the mineralized component of bone. Bone mobilization is achieved through the acidifying molecules, carbonic anhydrase II (CAII), an electrogenic H^+ATPase and a charge-coupled Cl^- channel (FIG. 2). Cathepsin K mediates bone organic matrix degradation. M-CSF, macrophage colony stimulating factor; RANK, receptor activator of NFκB.

Osteopetrosis. The increase in bone mass in individuals with osteopetrosis is accompanied by the loss of distinction between the BONE-MARROW SPACE and the CORTEX. Among the most notable cases of human osteopetrosis that helped to determine osteoclast ontogeny was the first cure by bone-marrow transplantation, which involved a female recipient and male donor[2]. This transgender transplant allowed the donor cells to be tracked, which enabled the haematological ontogeny of the osteoclast to be examined. Ultimately, the osteoclast has been found to be a multinucleated member of the monocyte/macrophage family[3] (FIG. 1).

The 'malignant' form of osteopetrosis is inherited as an autosomal recessive trait, whereas the 'benign' form is generally autosomal dominant and occasionally autosomal recessive[4]. The benign form is typified by the presence of dysfunctional osteoclasts. Similarly, the malignant phenotype can be caused not only by arrested osteoclast function, but also by failed osteoclast recruitment. The subset of osteopetroses that is caused by arrested osteoclastogenesis can be further subdivided into osteoclast autonomous and non-autonomous forms. Osteoclast-autonomous forms are those in which the molecular defect is resident in the osteoclast or its precursor. Osteoclast-non-autonomous forms represent those in which the molecular defect is present in cells that support osteoclast precursor differentiation or function of the mature resorptive cell.

Osteoporosis. Osteoporosis is a family of disorders in which systemic bone mass is sufficiently reduced to place the patient at risk of spontaneous fracture. Increased activity of individual osteoclasts or an increase in the overall number of osteoclasts can cause osteoporosis. Less commonly, abnormally high bone degradation that is caused by osteoclast gain of function can be localized to OSTEOLYTIC lesions such as those found in individuals with Paget disease of bone (PDB) or familial expansile osteolysis (FEO) (also known as hereditary expansile polyostotic osteolytic dysplasia or HEPOD). Most forms of adult osteoporosis represent alterations in the equilibrium between osteoclast and osteoblast recruitment in the bone-remodelling process (BOX 1).

BONE-MARROW SPACE
The cavity within bone that contains the bone marrow.

CORTEX
The densest portion of bone, which comprises approximately 80% of the skeletal mass and is an important structural component.

OSTEOLYTIC
The degradation of bone in pathological conditions.

Arrest of osteoclastogenesis

Failed osteoclast recruitment leads to osteopetrosis. So, it is through studies of this genetic disease, which is caused by the arrest of osteoclastogenesis, that we have gained much of our knowledge of the fate of the osteoclast and the genes that control it. Mutations in genes that encode proteins that are involved directly in early osteoclast precursor differentiation and commitment to the osteoclast fate have provided insights into osteoclastogenesis.

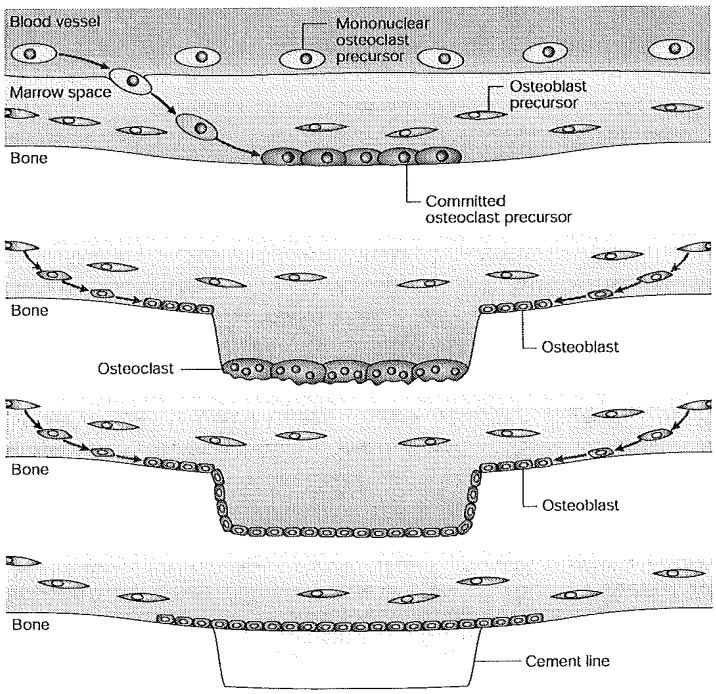
Mutations that influence early osteoclastogenesis. The molecule that mediates the earliest established event in osteoclastogenesis is the ETS domain transcription factor PU.1, which is encoded by the spleen focus forming virus (SFFV) proviral integration oncogene *PU.1* (*SPI1*) locus. *PU.1*$^{-/-}$ mice have osteoclast-deficient osteopetrosis, which reflects a failure to generate bone-marrow macrophages[5]. However, *PU.1*$^{-/-}$ fetal liver cultures can generate cells that express early monocytic characteristics[6] (FIG. 1). So, although PU.1 is the earliest known molecule to affect osteoclastogenesis, earlier acting molecules might remain to be discovered.

Much of the impact that PU.1 exerts on osteoclastogenesis reflects its interplay with the microphthalmia-associated transcription factor (MITF) family of proteins, which include TFE3, TFEB, TFEC and MITF[7,8], the last of which is encoded by the *microphthalmia* (*mi*) locus in mice. The three known mutations in the MITF family that lead to the arrest of osteoclastogenesis all involve MITF.

Box 1 | **Bone remodelling**

Bone remodelling is a continuous process that is responsible for normal bone turnover. The process is initiated by the recruitment of HAEMATOPOIETIC osteoclast precursors to bone, where they mature into resorptive POLYKARYONS. The differentiated osteoclasts generate a resorption lacunae ('pit') that is ~50μm deep, then are replaced by osteoblasts that deposit new bone in the cavity. The boundaries of the resorption lacunae are permanently marked by a METACHROMATIC CEMENT LINE.

When the skeleton is in balance, the amount of new bone deposited is equal to that previously resorbed. In states of net bone loss, such as osteoporosis, more bone is resorbed than is subsequently deposited.



MITF$^{mi/mi}$ is among the earliest recognized forms of murine osteopetrosis[9,10]. *mi/mi* osteoclasts fail to multinucleate or form ruffled membranes[11]. Failure of *mi/mi* mice to generate mature osteoclasts is linked to the fact that MITF targets genes that are required for osteoclast differentiation, such as those that encode tartrate-resistant acid phosphatase (TRAP) and carbonic anhydrase II (REFS 7,12). As *mi/mi* mice have abundant macrophages[8,11], MITF must affect the osteoclastogenic process later than PU.1 (FIG. 1). A reasonable hypothesis holds that the paucity of multinucleated osteoclasts in *mi/mi* mice is caused by the accelerated death of these terminally differentiated cells. In fact, *Bcl2* is also a MITF target gene, as *mi/mi* osteoclasts contain greatly diminished levels of the anti-apoptotic molecule[13]. Supporting the theory that a significant component of the *mi/mi* skeletal phenotype reflects accelerated apoptosis, *Bcl2*$^{-/-}$ mice are also severely osteopetrotic[13].

The *Mi*^{or} mutation, which is close to the *mi* mutation, lies in the exon that encodes the basic component of MITF[14–16]. Despite the proximity of *Mi*^{or} and *mi* mutations, the *Mi*^{or}/*Mi*^{or} phenotype is substantially less severe than that of *mi/mi*[17]. However, both MITF^{mi} and MITF *Mi*^{or} fail to bind the MITF E-box DNA recognition sequence[14]. These mutant molecules therefore sequester other MITF family members that, independent of MITF, have the capacity to stimulate osteoclastogenesis and alleviate osteopetrosis. The relatively unaffected *mib* rat, which is completely devoid of MITF, continues to express non-sequestered TFE3, which itself has the capacity to transactivate osteoclastogenic genes[15,16]. So, in contrast to the pronounced osteopetrosis of the *mi/mi* mouse, *mib/mib* osteoclasts can resorb mineralized matrix[18].

Macrophage colony stimulating factor (M-CSF) is expressed by osteoblasts and their precursors as a soluble and membrane-bound protein. In combination with RANKL, both forms are sufficient to promote the osteoclast differentiation of isolated monocytic precursors[19] (FIG. 1). The pivotal role of M-CSF in osteoclastogenesis was established by studies of the *op/op* mouse, which lacks functional M-CSF and so has osteoclast-deficient osteopetrosis[20]. Curiously, the skeletal lesion of the *op/op* mouse spontaneously resolves with age. It is now apparent that this resolution reflects the progressive expression of compensatory cytokines, specifically GM-CSF[21] and VEGF[22].

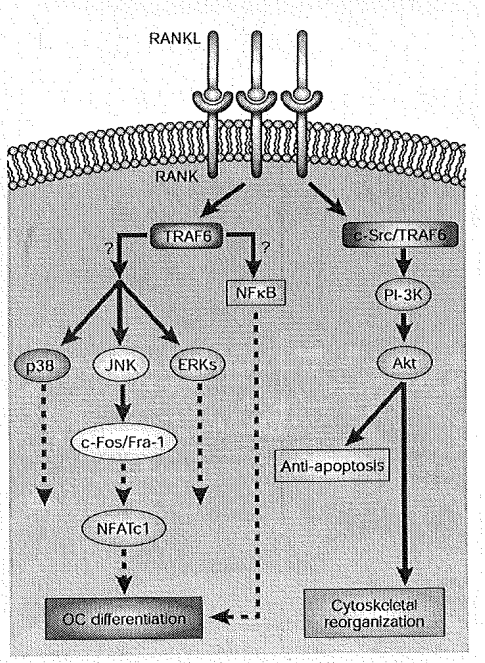
M-CSF promotes proliferation and survival of osteoclast precursors. The cytokine also stimulates activities of the mature resorptive cell such as spreading, motility and cytoskeletal organization[23]. These events involve the association of c-Src and phosphatidylinositol-3 kinase (PI-3K) with the M-CSF receptor c-Fms[23,24]. Because c-Fms is expressed on osteoclast precursors, deletion of its gene results in osteoclast-autonomous and osteoclast-deficient osteopetrosis[25].

HAEMATOPOIETIC
Giving rise to the cellular elements of the blood, such as white blood cells, red blood cells and platelets.

POLYKARYON
A multinucleated cell.

METACHROMATIC CEMENT LINE
A histological marker that delineates the depth of a remodelling site.

Box 2 | **Key elements of RANK signalling**



Binding of RANKL to RANK leads to receptor trimerization. This is followed by the recruitment of adaptor molecules, including TRAF6, which interact with c-Src to stimulate the PI-3K/Akt axis, which regulates both the osteoclast (OC) cytoskeleton and apoptosis. The detailed pathways that lead to activation of the three MAPKs and the various components of the NFκB complex are only partially defined. Important downstream regulators of osteoclast formation include c-Fos, Fra-1 and nuclear factor activated T cells c1 (NFATc1). ERKs, extracellular receptor kinases; JNK, jun kinase; NFκB, nuclear factor κB; PI-3K, phosphatidylinositol 3 kinase; RANK, receptor activator of NFκB; RANKL, RANK ligand; TRAF6, tumour necrosis factor receptor associated factor 6.

c-Fms is a receptor tyrosine kinase the autophosphorylation of which recruits essential adaptor molecules such as c-Src. Using a chimaera consisting of the intracellular and transmembrane domains of c-Fms and the extracellular domain of the erythropoietin receptor, we established that two tyrosine residues in the c-Fms cytoplasmic domain regulate osteoclast generation and/or function[26]. Tyr 807, the associated adaptor proteins of which are unknown, is essential for osteoclast differentiation but not function, whereas Tyr 559, which recognizes Src family members, is required for both phenomena.

Commitment to the osteoclast fate. Osteoclast precursors are monocyte/macrophages, but what prompts these cells to commit to the osteoclast fate was until recently unknown. Progress was made when Suda and colleagues generated osteoclasts *in vitro* for the first time. They showed that the process required contact with osteoblasts or their precursor bone-marrow stromal cells[3]. However, the molecular mechanism underlying this event remained unresolved until it was shown that RANKL (receptor activator of the NFκB ligand), which is encoded by *Tnfsf11*, impels macrophages along the osteoclastogenic pathway[27,28].

RANKL, a member of the tumour necrosis factor superfamily, is most abundantly expressed as a cell-surface protein by bone-marrow stromal cells. It interacts with its receptor RANK (which is encoded by *Tnfrsf11a*) on macrophages and mature osteoclasts (FIG. 1; BOX 2). Both *Tnfsf11*-knockout mice and mice in which *Tnfrsf11a* has been deleted ($RANK^{-/-}$), fail to generate osteoclasts and are severely osteopetrotic[27,29] (see Note added in proof). However, *Tnfrsf11a*-knockout mice are rescued by RANK-expressing haematopoietic cells, which indicates that RANK is an osteoclast-autonomous protein[29].

The discovery that RANKL is pivotal to osteoclastogenesis followed the finding of osteopetrosis in mice that overexpressed a molecule subsequently named osteoprotegerin (OPG)[30]. OPG, which is encoded by *Tnfrsf11b*, is also expressed by osteoblasts and their precursors[31]. OPG is secreted and competes with RANK as a soluble 'decoy receptor' for RANKL[30]. *Tnfrsf11b*-knockout mice have profound osteoporosis that arises from increased osteoclast number and activity[32].

RANK engagement by RANKL activates all three MAPK pathways as well as PI-3K and the NFκB family of transcription factors[33] (BOX 2). RANK promotes signalling by recruiting adaptor molecules after ligand-induced trimerization. Among such adaptors are the six TNF receptor-associated factors (TRAFs). RANK binds TRAF1, 2, 3 and 5 at its carboxyl terminus and TRAF6 at a more membrane-proximal site[34,35]. Although TRAF6 is essential for the organization of the osteoclast cytoskeleton[36], its impact on osteoclastogenesis is controversial. Specifically, two laboratories independently generated $Traf6^{-/-}$ mice with distinct phenotypes. In one case, the mutant mice have abundant, albeit dysfunctional, osteoclasts that are abnormal in appearance and lack both attachment zones and ruffled membranes, and suffer from severe osteopetrosis[37]. The other $Traf6^{-/-}$ strain is also osteopetrotic but is devoid of osteoclasts[38]. Although this problem has not yet been resolved, the fact that, based on the crystal structure of the RANK sequence recognizing TRAF6, a cell-permeable peptide arrests osteoclastogenesis *in vitro*[39], supports the idea that the adaptor molecule is essential for osteoclast differentiation.

The realization that RANKL is the main osteoclastogenic cytokine was one of the most compelling discoveries in the exploration of osteoclast development. Among the most important consequences of this observation is the capacity to generate pure populations of osteoclasts and their committed precursors from isolated macrophages. The abundance of these homogeneous cells allows meaningful evaluation of their biology *in vitro*. Knowledge of the global effect of RANKL on osteoclast biology came with the appreciation that the molecule not only prompts differentiation of its precursors, but also stimulates resorptive activity and prolongs the lifespan of the mature polykaryon[40].

Transcriptional influences of RANKL. The RANKL interaction with RANK leads to activation of many osteoclastogenic transcription factors, which include NFATc1. This is important because when NFATc1 is expressed in precursor cells it prompts them to undergo osteoclastogenesis, even in the absence of RANKL[41,42].

ANKYRIN REPEATS
A repeating amino-acid motif that mediates protein–protein interactions.

RANKL also influences another important pathway: that of nuclear factor κB (NFκB). Deletion of NFκB causes osteoclast-autonomous osteopetrosis, which is characterized by the absence of multinucleated bone-resorbing cells[43]. Macrophages are present in abundance, which indicates that NFκB might function later than PU.1 in the osteoclastogenic scheme (FIG. 1). Although there is little question that NFκB is pivotal to osteoclastogenesis, its impact on mature osteoclasts is unresolved[44,45].

NFκB activity is regulated by the IκB family of inhibitors, which use ANKYRIN REPEATS to bind and retain NFκB dimers in the cytosol. NFκB-activating cytokines, such as RANKL, TNFα and IL-1, rapidly initiate the classical NFκB pathway through the degradation of IκBα, which is the predominant IκB, so releasing active NFκB into the nucleus. As expected, the overexpression of IκBα blocks this classical pathway and inhibits osteoclastogenesis[46]. Also, recent studies describe an alternative NFκB pathway that is active in osteoclast precursors, which depends on NFκB inducing kinase (NIK) (D. Novack *et al.*, unpublished observations).

RANKL also induces the expression of c-Fos, a member of the AP-1 family of transcription factors, which partners with c-Jun[47] to have an essential role in osteoclastogenesis. *Fos*$^{-/-}$ mice are severely osteopetrotic and lack osteoclasts[48]. Interestingly, the number of tissue macrophages is increased in these mutants. These results, coupled with data showing that overexpression of c-Fos in monocytic cells prevents their differentiation into DENDRITIC CELLS[49], indicate that c-Fos directs monocytic precursors along an osteoclastogenic pathway and away from macrophage development.

Fra1, a member of the Fos family of proteins, is itself induced by c-Fos[50]. Overexpression of *Fosl1*, the gene that encodes Fra1, completely rescues the *Fos*$^{-/-}$ osteopetrotic mouse[51]. Furthermore, insertion of *Fosl1* into the *Fos* locus yields normal mice in a gene-dose-dependent fashion[51]. Because Fra1 lacks a transactivation domain it cannot bind DNA, and so its effect on osteoclastogenesis probably reflects its function as an accessory transcriptional molecule.

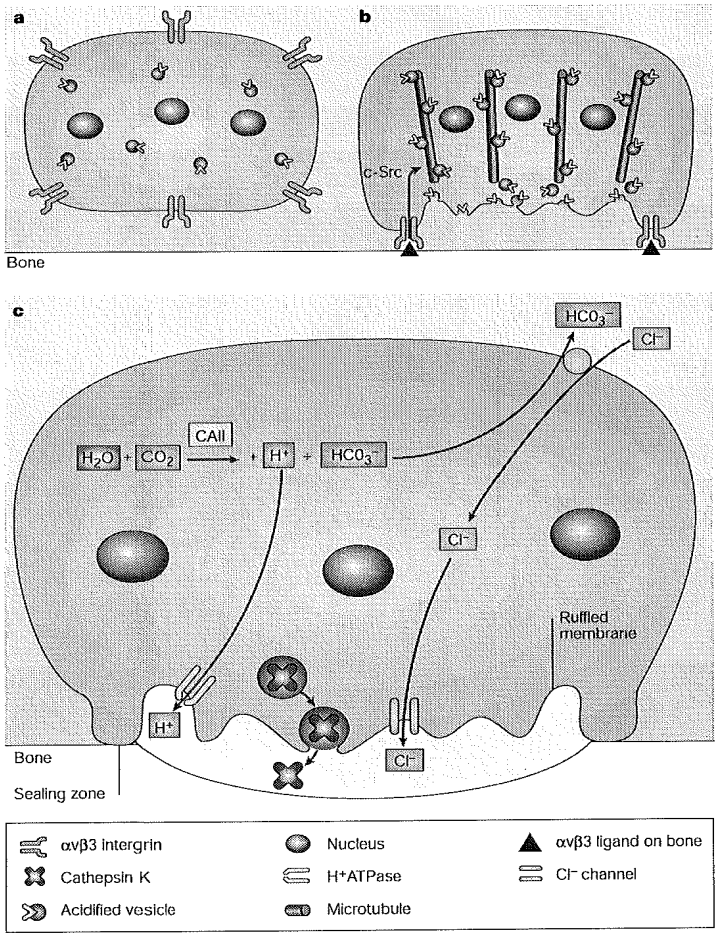


Figure 2 | **Regulation of the resorptive activity of osteoclasts. a** | The osteoclast, when not attached to bone, contains many diffusely-distributed acidified vesicles that express an electrogenic H⁺ATPase. Similarly, the αvβ3 integrin is expressed randomly on the plasma membrane. **b** | On attachment to bone, the integrin moves to the matrix-apposed membrane and transmits c-Src-dependent polarization signals, which then prompt the acidifying vesicles to associate with microtubules. The vesicles move along the microtubules and insert into the bone-apposed membrane in an exocytic fashion, to form the complex infoldings of the osteoclast resorptive organelle (the ruffled membrane). Insertion of the acidified vesicles into the ruffled membrane delivers the H⁺ATPase to the resorptive organelle. **c** | Bone degradation is initiated by formation of an actin-rich sealing zone that isolates the resorptive microenvironment from the general extracellular space. Protons that are generated under the influence of carbonic anhydrase II (CAII) are transported into the microenvironment through the ruffled-membrane-residing H⁺ATPase, bringing the ambient pH to ~4.5, which mobilizes the mineral component of bone. Intracellular pH homeostasis is maintained by exchange of bicarbonate for chloride ions. The chloride ions entering the cell are secreted into the resorptive microenvironment through an anion channel, charge coupled to the H⁺ATPase. Cathepsin K residing in vesicles is secreted in an exocytic process into the resorptive space and degrades the organic matrix of bone.

Arrest of osteoclast function

As well as providing insights into the molecules that regulate osteoclast differentiation, studies of osteopetrosis have helped to clarify the mechanisms by which mature osteoclasts degrade bone (FIG. 2). In contrast to the osteoclast-deficient family, the osteopetroses that result from failed osteoclast function are characterized by the presence of osteoclasts, albeit dysfunctional, and all known examples of these mutants represent osteoclast-autonomous disorders. Importantly, all patients with osteopetrosis whose molecular defect has been defined have abnormalities of osteoclast function.

Bone recognition and attachment. Bone degradation and terminal osteoclastogenesis require physical contact between the osteoclast and its precursor, respectively, and bone matrix. So, molecules by which the osteoclast recognizes and attaches to bone are essential for resorption. It is now clear that the integrin αvβ3 is central to osteoclast/bone recognition, because mice with the *β3* gene knocked out have dysfunctional osteoclasts and become progressively osteopetrotic[52].

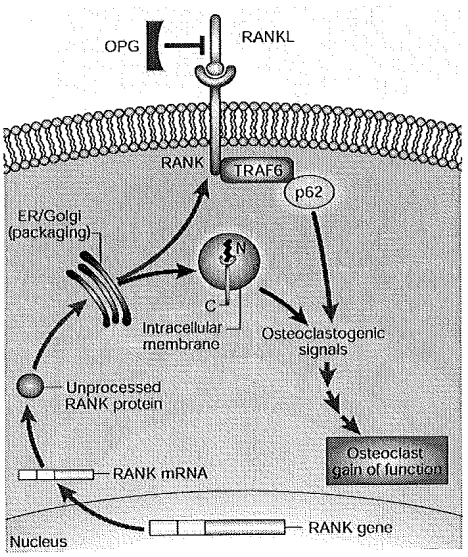


Figure 3 | **Mutations that lead to osteoclast gain of function.** As the RANK/RANK ligand (RANKL) signalling pathway is central to osteoclast formation and function (BOX 2), genetic mutations that result in its augmentation will enhance bone resorption. Mutations in three different proteins are known to augment osteoclast function. First, deletion of, or mutations in, the gene that encodes osteoprotegerin (OPG), which is the decoy receptor for RANKL, will increase RANK signals without altering levels of RANKL. Second, several mutations in the ubiquitin binding domain of p62, which is an adaptor that stimulates activation of the nuclear factor κB (NFκB) pathway, also stimulate osteoclastogenesis without the need for increased extracellular RANKL. Third, tandem duplication in the first exon of the RANK gene (yellow rectangles), which results in the presence of a further five, six or nine amino acids in the signal peptide (red chevron), lead to the retention of the unprocessed receptor in the cell (red arrows), rather than its insertion in the plasma membrane (blue arrow). The internal receptor is auto-activated by undefined mechanisms, thereby producing signals that lead to the generation of greater numbers of more active osteoclasts. RANK, receptor activator of nuclear factor κB; TRAF6, tumour necrosis factor receptor associated factor 6.

DENDRITIC CELLS
Phagocytic antigen-presenting cells that trigger adaptive immunity to a wide range of pathogens.

FOCAL ADHESIONS
Sites at which cells make contact with their substratum, which contain clusters of integrins with their extracellular parts bound to extracellular matrix proteins and their intracellular parts attached to actin filaments.

PLATELETS
Fragments of megakaryocytes that circulate in all mammals and regulate blood coagulation.

PROTEASOME
A large intracellular complex that degrades proteins to which ubiquitin has been added by a process that requires ATP.

RENAL TUBULAR ACIDOSIS
Low blood pH owing to the failure of renal tubules to regulate plasma bicarbonate concentration.

CEREBRAL CALCIFICATION
The deposition of calcium salts in the brain, which is typically identified by imaging techniques.

Interestingly, small molecule inhibitors of the αvβ3 integrin are promising anti-osteoporotic drugs[53] and such agents are in clinical trials.

In the bone-adherent osteoclast, αvβ3 associates with filamentous actin in a punctate complex that is known as the podosome, a structure akin to, but distinct from, FOCAL ADHESIONS in other cells[54] (FIG. 2b). αvβ3 recognizes proteins in the bone matrix that contain the Arg-Gly-Asp (RGD) amino-acid motif. Osteoclasts that lack αvβ3 still attach to bone but fail to organize their actin cytoskeleton into a sealing zone and have abnormal ruffled membranes[52]. So, the primary function of the integrin might actually be to transmit matrix-derived signals into the interior of the cell. This defect in cytoskeletal organization in αvβ3-deficient osteoclasts reflects the failure to activate the small GTPases, Rho and Rac (see Note added in proof). The β3 integrin subunit is expressed in PLATELETS as well as osteoclasts. Some *β3* mutations (such as that causing Glanzmann thrombasthenia), affect both platelet and osteoclast function, whereas others are platelet specific[55].

Paradoxically, although the number of mature osteoclasts that are derived from $\beta3^{-/-}$ macrophages is diminished in culture, $\beta3^{-/-}$ osteoclasts are increased *in vivo*[52]. High M-CSF levels in these mice induce c-Fos expression and rescue osteoclastogenesis[56]. Although M-CSF can substitute for αvβ3 in osteoclastogenesis (FIG. 1), the integrin is essential for osteoclast function (FIG. 2).

c-Src and Pyk2 are also involved in the podosomal signalling complex[57,58]. Inhibition of Pyk2 decreases osteoclast formation and function *in vitro*[59]. Ligand-bound αvβ3 activates Pyk2, but β3-deleted macrophages can also activate Pyk2, which indicates that αvβ3 is not an absolute requirement for phosphorylation (see Note added in proof). In any event, generation of the αvβ3-associated signalling complex seems to be initiated by adhesion-dependent phosphorylation of Pyk2, which leads to the activation of, and association with, c-Src[60].

Activated c-Src binds to and phosphorylates c-Cbl, which in turn inhibits c-Src kinase activity and matrix adhesion. c-Cbl, an E3 ubiquitin ligase that is also involved in osteoclast recruitment[61,62], polyubiquitylates c-Src, which probably leads to degradation of the Pyk2/c-Src/c-Cbl complex in the PROTEASOME[60]. So, generation of this heterotrimer might regulate podosomal assembly and disassembly, which is essential for the cyclic events of osteoclast migration and bone resorption (BOX 1). Despite *in vitro* documentation of the importance of αvβ3, c-Src, Pyk2 and c-Cbl in osteoclast function, only mice that lack the integrin[56] or c-Src[63] have been shown to have a substantial bone phenotype. Therefore, if Pyk2 and c-Cbl are important to the osteoclast *in vivo*, there are compensatory mechanisms that function in their absence. Studies in knockout mice confirm the importance of c-Src to osteoclast function[64,65] and its functional relationship with αvβ3 (REF. 66; FIG. 2b).

Ion transport. Once it is attached to bone, the osteoclast acidifies the isolated microenvironment at the cell–bone interface. This event is central to bone degradation as it mobilizes minerals, thereby exposing the organic matrix. Proton generation is initiated by the activity of carbonic anhydrase II (CAII), followed by polarized extracellular transport through an H^+ATPase similar to that of the clathrin-coated vesicle[67] (FIG. 2c). The bicarbonate ions that are generated by CAII are exchanged for chloride at the anti-resorptive surface of the osteoclast, thereby normalizing the intracellular pH. Electroneutrality is maintained, in turn, by a ruffled-membrane-residing chloride channel (reviewed in REF. 68).

Families that fail to express CAII develop osteopetrosis with a relatively benign phenotype, which is typically accompanied by RENAL TUBULAR ACIDOSIS and CEREBRAL CALCIFICATION[69]. Mutations of the osteoclast electrogenic

proton pump, particularly its a3 (116 kDa) transmembrane subunit, account for most of the documented cases of human malignant osteopetrosis[70–72]. Furthermore, the osteopetrotic lesion of *oc/oc* mice is caused by mutations in the same subunit[73]. These mice, and those in which the a3 subunit gene *Atp6i* (*Tcirg1*) has been deleted[74], have normal acid-transporting capacity in other cells, which might reflect redundant mechanisms or specific a3 expression in the osteoclast. Because proton-pump-deficient osteopetrosis represents an osteoclast-autonomous defect, it is responsive to bone-marrow transplantation, and patients with this abnormality probably represent the bulk of those cured in this way.

Once the osteoclast interacts with the bone surface, its H^+ATPase is transported in c-Src-containing vesicles towards the matrix-apposed plasma membrane through a polarization signal that is probably mediated through the $\alpha v\beta 3$ integrin[75]. The acidifying vesicles insert into the resorptive plasmalemma, mediated by small GTPases (N. Pavlos *et al.*, unpublished observations). It is this insertion of proton-pump-containing vesicles that results in the expanded and complex structure of the ruffled membrane. Mutations of either c-Src[64] or the electrogenic proton pump[71] prevent ruffled-membrane formation, which indicates their essential role in its development.

Because bone resorption involves massive extracellular proton transport, parallel anion conductance is needed to maintain intra-osteoclastic electroneutrality. Mice and humans that lack the CIC-7 chloride channel, which is expressed abundantly in the ruffled membrane[76], develop severe osteopetrosis[77]. These mice have many osteoclasts that cannot acidify the resorptive microenvironment and hence fail to resorb bone.

Degradation of organic matrix. Acidification of the resorptive microenvironment mobilizes the mineralized component of bone, thereby exposing its organic phase. Given this highly acidic environment, the degradation of the exposed organic matrix requires a collagenolytic enzyme with a low pH optimum, in this case, cathepsin K (FIG. 2c). Cathepsin K was identified as the osteoclast collagenolytic enzyme when it was shown that mutations in this enzyme caused the SCLEROSING bone disorder pycnodysostosis[78]. Osteoclasts of pycnodysostotic patients, as well as osteopetrotic *Ctsk*-knockout mice, generate ruffled membranes and mobilize bone mineral, but fail to normally degrade the exposed collagen fibres that accumulate in the resorptive microenvironment[79,80].

The *Ctsk* gene is transactivated by MITF and its dimerizing partner TFE3. So, the lysosomal protease is deficient in *mi/mi* osteoclasts[81]. Cathepsin K is generated as a ZYMOGEN, but activated in the osteoclast as it progresses from the Golgi to the ruffled membrane for secretion. Such processing begins as the osteoclast approaches the bone surface. The bone-attached cell expresses only the mature form of the enzyme, which indicates that matrix-derived signals might induce intracellular cathepsin K maturation[82]. Uniquely among mammalian proteinases, cathepsin K targets various sites inside and outside the collagen helix[83]. Importantly, cathepsin K is a promising therapeutic target, as it has been shown that small-molecule inhibitors prevent experimental post-menopausal osteoporosis[84,85].

SCLEROSING
The process of pathologically enhancing bone mass, which is typically detected by radiography or densitometry.

ZYMOGEN
An inactive form of an enzyme, which requires proteolytic processing for activation.

Osteoclast gain of function

Loss-of-function mutations that cause osteopetrosis in both humans and mice provide insights into the mechanisms by which osteoclasts are recruited to and resorb bone. Also, the discovery of mutations in several proteins that modulate the RANKL signalling pathway has elucidated the pathogenetic mechanisms of several diseases that are characterized by enhanced osteoclast number and/or activity and low bone mass (FIG. 3).

Mutations in RANK. The expansile osteolysis, FEO, is a disorder of rapid bone loss that is inherited as a highly penetrant autosomal-dominant trait. Early-linkage studies have mapped FEO to the region on chromosome 18q21.2-21.3 that contains *TNFRSF11A*[86]. Subsequently, affected individuals in three FEO families were shown to have an identical 18-bp tandem duplication in exon 1 of *TNFRSF11A*, which encodes the RANK signal peptide[87].

PDB is a common, often multifocal, skeletal disorder that also reflects enhanced osteoclast recruitment and function. Most PDB (~70%) occurs sporadically and is associated with viral infections, which has led to the hypothesis that such agents have a causative role. A small proportion of cases are attributable to mutations in *TNFRSF11A*, *TNFRSF11B* (the gene that encodes the decoy RANKL receptor OPG) and p62, which is an adaptor linked to RANK signalling (see below). So, a significant number of further mutations that result in PDB remain to be discovered. As in FEO, a Japanese family with early-onset (familial) PDB has a tandem 27-bp duplication that encodes the same mutant RANK signal peptide that was reported in FEO, plus three extra amino acids[88]. Because this particular duplication was established in only one of the four PDB families studied, further mutations on chromosome 18, possibly in other *TNFRSF11A* exons, might exist.

Expansile skeletal hyperphosphatasia (ESH) is a rare autosomal dominant disorder[89] in which RANK once again contains an elongated signal peptide. The expanded genomic sequence, also a tandem duplication in exon 1 of *TNFRSF11A*, but comprising 15 bp, lacks only the first three bases of the 18-bp FEO duplication and hence one amino acid. Although the clinical manifestations of ESH are caused by accelerated bone remodelling, the absence of large osteolytic lesions in the major long bones indicates that the clinical phenotype might differ from 'classical' FEO.

Presence of the extra amino acids in FEO, as well as chromosome 18 mutations that lead to early-onset-PDB, arrest cleavage of the RANK signal peptide, which attenuates plasma-membrane insertion of the receptor and sequesters it intracellularly[87]. Because these *TNFRSF11A* mutations are associated with enhanced osteoclastogenesis in FEO and early-onset PDB, it seems that RANK can signal before its cell-surface expression. Support for this

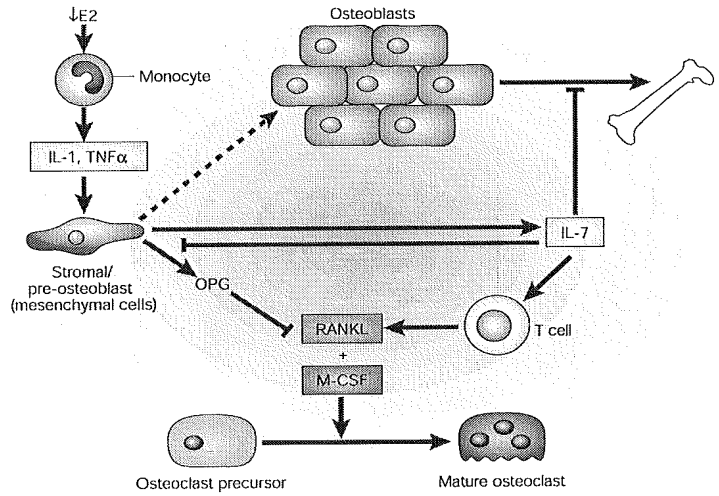


Figure 4 | **Role of T cells and IL-7 in oestrogen-mediated bone loss *in vivo*.** A lack of oestrogen (E2) results in the release of interleukin 1 (IL-1) and tumour necrosis factor-α (TNFα) by circulating monocytes. These cytokines then target mesenchymal cells and stimulate them to release interleukin 7 (IL-7), which, in turn, acts on both osteoblasts and T cells. Overall, IL-7 release from T cells as a consequence of gonadal failure leads to the loss of bone mass by two reciprocal pathways: the suppression of bone formation and the activation of bone resorption. The lymphokine both suppresses osteoblast function and stimulates production of the pro-osteoclastogenic cytokines macrophage colony stimulating factor (M-CSF) and receptor activator of nuclear factor κ-B ligand (RANKL), while simultaneously decreasing expression of osteoprotegerin (OPG), which is the decoy receptor for RANKL. As a consequence of the latter changes, the net osteoclastic activity is increased. Also, monocyte-derived IL-1 and TNFα synergize with RANKL to further increase bone loss (not shown). Reproduced with permission from REF. 126.

hypothesis is provided by the fact that NFκB activation is enhanced in cells that are transfected with the RANK mutants that characterize FEO and early-onset PDB[87]. Increased NFκB activity, which presumably derives from heightened RANK-derived signalling, explains much of the stimulated osteoclastogenesis that causes these skeletal diseases. So, FEO, early-onset PDB and probably ESH, reflect auto-activated RANK, in which the signal peptide resists cleavage in the secretory pathway. What remains unclear is if such subtle changes in amino-acid sequence of the signal-peptide region of RANK cause distinct diseases of varying severity.

Mutations in osteoprotegerin. Although these findings represent one mechanism by which RANK signalling is augmented, others include events that are upstream and downstream of the receptor. For example, Navajo patients with autosomal recessive juvenile PDB have complete deletion of *TNFRSF11B*, which is located on chromosome 8q24.2 (REF. 90). As expected, serum levels of OPG are undetectable, whereas those of 'free' RANKL, which is presumably a biologically active molecule, are markedly increased[91]. So, OPG is central to normal bone remodelling in humans. The patients, from separate families, share identical break points that involve chromosome 8q24.2, which indicates familial PDB cases in the Navajo population might have a common origin and could have become more frequent owing to a FOUNDER EFFECT[91]. Furthermore, three siblings in an Iraqi family with familial PDB each have a homozygous 3-bp in-frame deletion in exon 3 of *TNFRSF11B*, which leads to the loss of an aspartame (D182) residue in OPG[92]. Unlike the Navajo patients, serum levels of OPG in these children are normal, but the protein lacking D182 does not block bone resorption. OPG D182 is in a region that is probably crucial for correct folding, which indicates that the loss of function in the mutant protein arises from its inability to bind, and hence to inactivate, RANKL.

Mutations in p62. Mutations in *TNFRSF11A* and *TNFRSF11B*, which are both involved in RANKL-RANK signalling, must represent only a small fraction of the individuals that suffer from familial or even sporadic PDB. In fact, other loci that are associated with typical PDB include 6p21.3 (PDB1), 18q23 (PDB2), 5q35–qter (PDB3) and 5q31 (PDB4)[93,94]. PDB3 localizes to a region on chromosome 5 that contains sequestosome 1 (*SQSTM1*), which encodes p-62 (REF. 93). *SQSTM1* in French-Canadian patients with PDB3 contains a C to T transversion mutation in exon 8 at position 1215, thereby replacing proline 392 with leucine. Only one severely affected individual is homozygous for the mutation. In fact, although the same *SQSTM1* P392L mutation exists in as many as half of the patients with familial PDB, it is also relatively common in those with sporadic disease. The familial disorder might also be characterized by a premature stop codon at position 396 in *SQSTM1* or a single mutation at the splice donor site in intron 7. The latter mutation leads to a protein that is truncated at position 390 (REF. 94). Because all these *SQSTM1* modifications occur between amino acids 384 and 434, this locus represents a potential preferred site for genetically-transmitted mutations and a hot spot for spontaneous mutations. Moreover, as all of the documented mutations fall within the ubiquitin recognition domain of p62 they might disrupt osteoclasts by a common mechanism[95].

p62 interacts with TRAF6 in the context of IL-1 and nerve growth-factor signalling[95,96]. The fact that TRAF6 is an essential component of RANK-mediated osteoclastogenesis raises the possibility that p62 is also involved in the process. The TRAF6/p62 interaction is thought to lead to the activation of NFκB. Therefore, it would be predicted that the P382L mutation in p62 should decrease osteoclast formation, which is dependent on the nuclear translocation of NFκB. In fact, the mutation leads to increased osteoclastogenesis as manifest by PDB. So, the role of p62 might differ in the context of RANK and the osteoclast. One possibility is that, when bound to the p62 ubiquitylation site, TRAF6 is degraded. Mutation of the site would again lead to enhanced signalling.

Mutations in SHIP. A more speculative mutation that might contribute to the pathophysiology of familial PDB concerns *SHIP* (*INPP5D*), a gene that encodes src homology (SH)-2 containing inositol-5′-phosphatase.

FOUNDER EFFECT
A gene mutation that is observed in high frequency in a specific population owing to its presence in a single ancestor or a small number of ancestors.

This enzyme removes 5′ phosphate from phosphatidylinositol 3,4,5-trisphosphate (PIP3), thereby producing functionally distinct phosphatidylinositol 3,4-bisphosphate (PIP2)[97]. As SHIP acts as a negative regulator of intracellular signalling, *SHIP* deletion, which is a loss-of-function event, results in a gain of function for those pathways that the enzyme inhibits[98]. For example, absence of the protein, expression of which is restricted to haematopoietic cells, results in myeloid and lymphoid cells that show hypersensitivity to a range of cytokines[99].

Mice that lack SHIP have low bone mass owing to prolonged survival and greater bone-resorptive capacity of their osteoclasts, which are morphologically indistinguishable from those of PDB[100]. Consistent with the inhibitory role of SHIP, osteoclast precursors that lack the enzyme are hypersensitive to M-CSF and RANKL. Furthermore, human *SHIP* maps to 18q23 (REF. 101), which is a documented locus for PDB, raising the possibility that mutations in *SHIP*, which undoubtedly increase bone resorption, might be the genetic defects in a subset of patients with PDB.

Oestrogen and the osteoclast

Post-menopausal osteoporosis results from alterations in cytokines, which, directly or indirectly, target osteoclasts and their precursors, thereby increasing the number and activity of bone-resorbing cells. What is the genetic evidence as to the identity of these cytokines, where are they produced and how does the post-menopausal decrease in oestrogen affect their expression?

As reviewed in REF. 102, a combination of naturally occuring human mutations plus the availability of mice that lack the two oestrogen receptors ERα and ERβ, has provided important insights into the role of oestrogen in bone metabolism. In summary, oestrogen is the sex steroid that is responsible for the regulation of human bone physiology in both males and females, with ERα being the dominant isoform that mediates the overall process. Also, studies of post-menopausal women[103,104] and mice[105–107] have clearly shown that reduced levels of oestrogen lead to higher levels of IL-1 and TNFα produced by monocytes, which indirectly promotes osteoclast precursor proliferation and survival[108,109] (FIG. 4).

However, as well as influencing osteoclastogenesis through its effect on production of the inflammatory cytokines by monocytes, oestrogen also acts through the cells of the lymphoid lineage. These cells also produce IL-1, TNFα and other proteins that modulate the resorptive response. Nude mice that lack T cells do not lose bone or develop the osteoporosis that occurs in wild-type animals following OOPHORECTOMY[110]. Also, knockout mice that lack the p55 TNFα receptor do not show T-cell induced osteoclast formation[110], which indicates that the interaction of TNFα with RANKL is crucial for osteoclastogenesis. Transplantation of bone marrow from wild-type mice into p55-TNFα-receptor –/– animals rescued these knockouts and so independently showed the crucial role of TNFα[111]. Finally, the adoptive transfer of wild-type T cells into nude mice, but not those lacking the *Tnf* gene, normalizes oophorectomy-induced bone loss[107].

OOPHORECTOMY
The surgical removal of the ovaries, which results in systemic oestrogen deficiency.

Immune recognition has a central role in the pathogenesis of post-menopausal osteoporosis. Increased T-cell number results from both enhanced proliferation and decreased apoptosis, which are events that are driven by the IFN-γ-stimulated expression of class II transactivator (CIITA). CIITA decreases the apoptosis of activated T cells through the modulation of FASL[112]. In summary, more TNFα-secreting T cells are produced and survive and the consequent higher levels of the inflammatory cytokine stimulate osteoclast formation, and hence, bone resorption. A recent seminal report shows that women who lack oestrogen have higher expression of the important osteoclastogenic molecule RANKL on both osteoblasts and lymphocytes[113]. This observation strongly supports earlier animal and *in vitro* human data that link the absence of sex steroid, RANKL and enhanced bone resorption.

However, the influence of inflammatory cytokines on osteoclastogenesis is not a simple one-way effect. IFNα also functions to negatively modulate RANK-mediated signalling. For example, LPS-induced osteoclastogenesis and bone resorption are blunted in IFNα-receptor-deleted mice[114]. *In vivo* data indicate that IFNα might arrest RANKL-induced activation of NFκB and JNK. Determining how the pro- and anti-osteoclastogenic properties of IFNα manifest themselves physiologically and pathologically presents an important challenge.

The IFNβ signalling pathway also affects osteoclastogenesis[115]. For example, mice that lack Ifnar1, which is a component of the receptor transducing Ifnα/β signals,

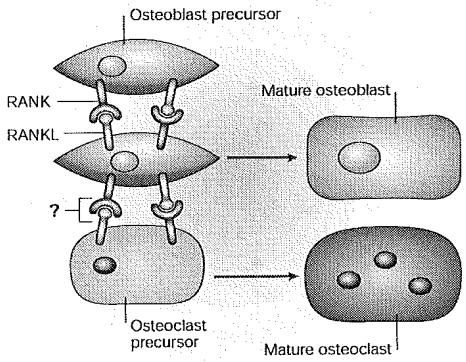


Figure 5 | **RANK ligand might induce osteoblast and osteoclast differentiation.** Osteoblast and osteoclast precursors express both receptor activator of nuclear factor κB (RANK) and RANK ligand (RANKL). RANKL, which resides in the plasma membrane of osteoblasts and their progenitors, binds to RANK on osteoclast precursors to promote osteoclast differentiation. The same ligand also activates RANK on osteoblast precursors, which prompts osteoblastogenesis. Although both RANK and RANKL are expressed by osteoclasts, the capacity of osteoclasts to directly promote osteoblastogenesis is not definitively established.

contain more osteoclasts than do their wild-type counterparts. Moreover, IFNβ inhibits M-CSF and RANKL-induced osteoclastogenesis, and *Ifnar1*$^{-/-}$ mice lacking Ifnar1 are osteoporotic. IL-6 might also participate in oestrogen-dependent bone loss. IL-6 production is enhanced in cultures of osteoblasts from mice that lack ovaries[116] and an anti-IL-6 antibody blocks oophorectomy-induced osteoclastogenesis[117]. On the basis of these observations, Yamaguchi and colleagues generated mice lacking the protein gp130, which is the common signalling transducer for members of the IL-6 cytokine subfamily, including IL-11, oncostatin M and leukaemia inhibitory factor, all of which might influence osteoclast formation. Surprisingly, the numbers of osteoclasts are increased in these animals, but the osteoblasts are normal[118]. So, although members of the IL-6 family of cytokines might have a role in osteoporosis that arises from oestrogen deficiency, they are not important for bone formation.

Finally, although other T cell and macrophage-derived cytokines, such as MIP-1, IL-10, GM-CSF, IL-4, IL-12 and IL-18, have also been reported to have a role in osteoclast formation and function[119–123], the genetic basis for these results and hence their *in vivo* relevance, is only partially delineated[120,123].

Conclusion

Over the past five years, we have learned a great deal about the mechanisms by which the osteoclast is formed and resorbs bone. Most of this progress has come about as a result of rapid advances in cell and molecular biology, which owe much to the explosion of information from genetically-based studies. We now have a clear picture as to the cytokines, enzymes and adhesion molecules, and the associated signal-transduction pathways, which are important for osteoclastogenesis and bone resorption. This level of understanding will result in the rational development of new drugs that can inhibit the generation and/or activity of this unique member of the monocyte/macrophage family.

Despite these advances, several key questions remain unanswered, one of which relates to the detailed mechanism of osteoclast polarization — a process that is central to its function. More important, however, is the issue of how bone degradation and formation are coupled in the remodelling process. Although it has been appreciated for some time that the two cell types engage in cross-talk, it is only more recently that the molecular basis of this process has been elucidated, with the main molecule apparently being RANKL. So, the observation that basal osteoclast formation requires contact between myeloid precursors and stromal cells is explained by the fact, that in most circumstances, RANKL is largely membrane bound. A greater surprise, based on as yet unpublished data from our laboratory, is that the same cytokine, which is resident on osteoblasts and their precursors, might have an important role in the activation of osteoblasts. Although speculative, it seems that at least one component of the long-elusive 'coupling factor' might be RANKL, the role of which in skeletal biology seems to go beyond being the main osteoclastogenic cytokine (FIG. 5). The definitive test of this speculative hypothesis would be to generate mice that lack RANK only in osteoblasts. These animals should have normal bone resorption, but decreased rates of bone formation.

Notes added in proof

A recent study by Faccio *et al.*[124] confirms that αvβ3-deficient osteoclasts cannot properly organize their cytoskeleton because the small GTPases, Rho and Rac, are not activated. These cells, however, activate Pyk2. Also, Kong *et al.*[125] showed that deletion of the *Tnfsf11* gene, which encodes murine RANKL, results in severe osteopetrosis; this finding is consistent with a role for the cytokine as the key osteoclast differentiation factor.

1. Blair, H. C., Teitelbaum, S. L., Ghiselli, R. & Gluck, S. Osteoclastic bone resorption by a polarized vacuolar proton pump. *Science* **245**, 855–857 (1989).
2. Coccia, P. F. *et al.* Successful bone-marrow transplantation for infantile malignant osteopetrosis. *N. Engl. J. Med.* **302**, 701–708 (1980).
3. Udagawa, N. *et al.* Origin of osteoclasts: mature monocytes and macrophages are capable of differentiating into osteoclasts under a suitable microenvironment prepared by bone marrow-derived stromal cells. *Proc. Natl Acad. Sci. USA* **87**, 7260–7264 (1990).
4. Sly, W. S. *et al.* Carbonic anhydrase II deficiency in 12 families with the autosomal recessive syndrome of osteopetrosis with renal tubular acidosis and cerebral calcification. *N. Engl. J. Med.* **313**, 139–145 (1985).
5. Tondravi, M. M. *et al.* Osteopetrosis in mice lacking haematopoietic transcription factor PU.1. *Nature* **386**, 81–84 (1997).
6. Henkel, G. W., McKercher, S. R., Leenen, P. J. M. & Maki, R. A. Commitment to the monocytic lineage occurs in the absence of the transcription factor PU.1. *Blood* **93**, 2849–2858 (1999).
7. Luchin, A. *et al.* Genetic and physical interactions between microphthalmia transcription factor and PU.1 are necessary for osteoclast gene expression and differentiation. *J. Biol. Chem.* **276**, 36703–36710 (2001).
8. Rehli, M., Lichanska, A., Cassady, A. I., Ostrowski, M. C. & Hume, D. A. TFEC is a macrophage-restricted member of the microphthalmia-TFE subfamily of basic helix–loop–helix leucine zipper transcription factors. *J. Immunol.* **162**, 1559–1565 (1999).
9. Walker, D. G. Bone resorption restored in osteopetrotic mice by transplants of normal bone marrow and spleen cells. *Science* **190**, 784–785 (1975).
10. Walker, D. G. Spleen cells transmit osteopetrosis in mice. *Science* **190**, 785–787 (1975).
11. Thesingh, C. W. & Scherft, J. P. Fusion disability of embryonic osteoclast precursor cells and macrophages in the microphthalmic osteopetrotic mouse. *Bone* **6**, 43–52 (1985).
12. Luchin, A. *et al.* The microphthalmia transcription factor regulates expression of the tartrate-resistant acid phosphatase gene during terminal differentiation of osteoclasts. *J. Bone Miner. Res.* **15**, 451–460 (2000).
13. McGill, G. G. *et al.* Bcl2 regulation by the melanocyte master regulator Mitf modulates lineage survival and melanoma cell viability. *Cell* **109**, 707–718 (2002).
14. Steingrimsson, E. *et al.* Molecular basis of mouse microphthalmia (*mi*) mutations helps explain their developmental and phenotypic consequences. *Nature Genet.* **8**, 256–263 (1994).
15. Weilbaecher, K. N. *et al.* Age-resolving osteopetrosis: a rat model implicating microphthalmia and the related transcription factor TFE3. *J. Exp. Med.* **187**, 775–785 (1998).
16. Moore, K. J. Insight into the microphthalmia gene. *Trends Genet.* **11**, 442–448 (1995).
17. Nii, A., Steingrimsson, E., Copeland, N. G., Jenkins, N. A. & Ward, J. M. Mild osteopetrosis in the microphthalmia-oak ridge mouse: a model for intermediate autosomal recessive osteopetrosis in humans. *Am. J. Pathol.* **147**, 1871–1882 (1995).
18. Cielinski, M. J. & Marks, S. C. Bone metabolism in the osteopetrotic rat mutation microphthalmia blanc. *Bone* **16**, 567–574 (1995).
19. Yao, G.-Q., Sun, B. H., Weir, E. C. & Insogna, K. L. A role for cell-surface CSF-1 in osteoblast-mediated osteoclastogenesis. *Calcif. Tissue Int.* **70**, 339–346 (2002).
20. Yoshida, H. *et al.* The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene. *Nature* **345**, 442–444 (1990).
21. Myint, Y. Y. *et al.* Granulocyte/macrophage colony-stimulating factor and interleukin-3 correct osteopetrosis in mice with osteopetrosis mutation. *Am. J. Pathol.* **154**, 553–566 (1999).
22. Niida, S. *et al.* Vascular endothelial growth factor can substitute for macrophage colony-stimulating factor in the support of osteoclastic bone resorption. *J. Exp. Med.* **190**, 293–298 (1999).
23. Insogna, K. L. *et al.* Colony-stimulating factor-1 induces cytoskeletal reorganization and c-src-dependent tyrosine phosphorylation of selected cellular proteins in rodent osteoclasts. *J. Clin. Invest.* **100**, 2476–2485 (1997).
24. Grey, A., Chen, Y., Paliwal, I., Carlberg, K. & Insogna, K. Evidence for a functional association between phosphatidylinositol 3-kinase and c-src in the spreading response of osteoclasts to colony-stimulating factor-1. *Endocrinology* **141**, 2129–2138 (2000).
25. Dai, X.-M. *et al.* Targeted disruption of the mouse colony-stimulating factor 1 receptor gene results in osteopetrosis, mononuclear phagocyte deficiency, increased primitive progenitor cell frequencies, and reproductive defects. *Blood* **99**, 111–120 (2002).

26. Feng, X. *et al.* Tyrosines 559 and 807 in the cytoplasmic tail of the m-*csf* receptor play distinct roles in osteoclast differentiation and function. *Endocrinology* **143**, 4868–4874 (2002).
27. Lacey, D. L. *et al.* Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell* **93**, 165–176 (1998).
Perhaps the most important study of osteoclastogenesis, in which RANKL (OPGL) is shown to be the main osteoclastogenic cytokine both *in vitro* and *in vivo*.
28. Yasuda, H. *et al.* Osteoclast differentiation factor is a ligand for osteoprotegenn/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. *Proc. Natl Acad. Sci. USA* **95**, 3597–3602 (1998).
29. Li, J. *et al.* RANK is the intrinsic hematopoietic cell surface receptor that controls osteoclastogenesis and regulation of bone mass and calcium metabolism. *Proc. Natl Acad. Sci. USA* **97**, 1566–1571 (2000).
30. Simonet, W. S. *et al.* Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. *Cell* **89**, 309–319 (1997).
This important paper identifies OPG as an anti-osteoclastogenic molecule the overexpression of which leads to osteopetrosis, and the administration of which prevents experimental post-menopausal osteoporosis.
31. Thomas, G. P., Baker, S. U. K., Eisman, J. A. & Gardiner, E. M. Changing RANKL/OPG mRNA expression in differentiating murine primary osteoblasts. *J. Endocrinol.* **170**, 451–460 (2001).
32. Bucay, N. *et al.* Osteoprotegerin-deficient mice develop early onset osteoporosis and arterial calcification. *Genes Dev.* **12**, 1260–1268 (1998).
33. Ross, F. P. RANKing the importance of measles virus in Paget's disease. *J. Clin. Invest.* **105**, 555–558 (2000).
34. Darnay, B. G., Ni, J., Moore, P. A. & Aggarwal, B. B. Activation of NF-κB by RANK requires tumor necrosis factor receptor-associated factor (TRAF) 6 and NF-κB-inducing kinase. *J. Biol. Chem.* **274**, 7724–7731 (1999).
35. Galibert, L. *et al.* The involvement of multiple tumor necrosis factor receptor (TNFR)-associated factors in the signaling mechanisms of receptor activator of NFκB, a member of the TNFR superfamily. *J. Biol. Chem.* **273**, 34120–34127 (1998).
36. Armstrong, A. P. *et al.* A RANK/TRAF6-dependent signal transduction pathway is essential for osteoclast cytoskeletal organization and resorptive function. *J. Biol. Chem.* **277**, 44347–44356 (2002).
37. Lomaga, M. A. *et al.* TRAF6 deficiency results in osteopetrosis and defective interleukin-1, CD40, and LPS signaling. *Genes Dev.* **13**, 1015–1024 (1999).
38. Naito, A. *et al.* Severe osteopetrosis, defective interleukin-1 signalling and lymph node organogenesis in TRAF6-deficient mice. *Genes Cells* **4**, 353–362 (1999).
39. Ye, H. *et al.* Distinct molecular mechanism for initiating TRAF6 signalling. *Nature* **418**, 443–447 (2002).
40. Burgess, T. L. *et al.* The ligand for osteoprotegerin (OPGL) directly activates mature osteoclasts. *J. Cell Biol.* **145**, 527–538 (1999).
41. Ishida, N. *et al.* Large scale gene expression analysis of osteoclastogenesis *in vitro* and elucidation of NFAT2 as a key regulator. *J. Biol. Chem.* **277**, 41147–41156 (2002).
42. Takayanagi, H. *et al.* Induction and activation of the transcription factor NFATc1 (NFAT2) integrate RANKL signaling in terminal differentiation of osteoclasts. *Dev. Cell* **3**, 889–901 (2002).
43. Franzoso, G. *et al.* Requirement for NFκB in osteoclast and B-cell development. *Genes Dev.* **11**, 3482–3496 (1997).
This study establishes the NFκB-signalling pathway as essential for osteoclastogenesis and shows that the event requires either the p50 or p52 subunit of the transcription complex.
44. Jimi, E. *et al.* Activation of NFκB is involved in the survival of osteoclasts promoted by interleukin-1. *J. Biol. Chem.* **273**, 8799–8805 (1998).
45. Miyazaki, T. *et al.* Reciprocal role of ERK and NFκB pathways in survival and activation of osteoclasts. *J. Cell Biol.* **148**, 333–342 (2000).
46. Abu-Amer, Y., Dowdy, S. F., Ross, F. P., Clohisy, J. C. & Teitelbaum, S. L. TAT fusion proteins containing tyrosine 42-deleted IκBα arrest osteoclastogenesis. *J. Biol. Chem.* **276**, 30499–30503 (2001).
47. David, J.-P., Sabapathy, K., Hoffmann, O., Idarraga, M. H. & Wagner, E. F. JNK1 modulates osteoclastogenesis through both c-Jun phosphorylation-dependent and-independent mechanisms. *J. Cell Sci.* **115**, 4317–4325 (2002).
48. Grigoriadis, A. E. *et al.* c-Fos: a key regulator of osteoclast-macrophage lineage determination and bone remodeling. *Science* **266**, 443–448 (1994).
49. Miyamoto, T. *et al.* Bifurcation of osteoclasts and dendritic cells from common progenitors. *Blood* **98**, 2544–2554 (2001).
50. Matsuo, K. *et al.* Fosl1 is a transcriptional target of c-Fos during osteoclast differentiation. *Nature Genet.* **24**, 184–187 (2000).
In this study, the authors establish that RANKL recruits osteoclasts in an AP-1 dependent manner in which c-Fos, which is required for osteoclastogenesis, induces the transcription of *Fosl1*.
51. Fleischmann, A. *et al.* Fra-1 replaces c-Fos-dependent functions in mice. *Genes Dev.* **14**, 2695–2700 (2000).
52. McHugh, K. P. *et al.* Mice lacking β3 integrins are osteosclerotic because of dysfunctional osteoclasts. *J. Clin. Invest.* **105**, 433–440 (2000).
The authors establish that the αvβ3 integrin is a candidate anti-osteoporosis therapeutic target, as mice with their β3 gene deleted have dysfunctional osteoclasts and develop osteopetrosis.
53. Engleman, V. W. *et al.* A peptidomimetic antagonist of the αvβ3 integrin inhibits bone resorption *in vitro* and prevents osteoporosis *in vivo*. *J. Clin. Invest.* **99**, 2284–2292 (1997).
54. Aubin, J. E. Osteoclast adhesion and resorption: the role of podosomes. *J. Bone Miner. Res.* **7**, 365–368 (1992).
55. Feng, X. *et al.* A Glanzmann's mutation of the β3 integrin gene specifically impairs osteoclast function. *J. Clin. Invest.* **107**, 1137–1144 (2001).
56. Faccio, R., Zallone, A., Ross, F. P. & Teitelbaum, S. L. c-Fms and the αvβ3 integrin collaborate during osteoclast differentiation. *J. Clin. Invest.* **111**, 749–758 (2003).
57. Sanjay, A. *et al.* Cbl associates with Pyk2 and Src to regulate Src kinase activity, αvβ3 integrin-mediated signaling, cell adhesion, and osteoclast motility. *J. Cell Biol.* **152**, 181–196 (2001).
58. Pfaff, M. & Jurdic, P. Podosomes in osteoclast-like cells: structural analysis and cooperative roles of paxillin, proline-rich tyrosine kinase 2 (Pyk2) and integrin αvβ3. *J. Cell Sci.* **114**, 2775–2786 (2001).
59. Duong, L. T. *et al.* Inhibition of osteoclast function by adenovirus expressing antisense protein-tyrosine kinase 2. *J. Biol. Chem.* **276**, 7484–7492 (2001).
60. Yokouchi, M. *et al.* Src-catalyzed phosphorylation of c-Cbl leads to the interdependent ubiquitination of both proteins. *J. Biol. Chem.* **276**, 35185–35193 (2001).
61. Lee, P. S. W. *et al.* The Cbl protooncoprotein stimulates CSF-1 receptor multiubiquitination and endocytosis, and attenuates macrophage proliferation. *EMBO J.* **18**, 3616–3628 (1999).
62. Wang, Y., Yeung, Y.-G. & Stanley, E. R. CSF-1 stimulated multiubiquitination of the CSF-1 receptor and of Cbl follows their tyrosine phosphorylation and association with other signaling proteins. *J. Cell. Biochem.* **72**, 119–134 (1999).
63. Soriano, P., Montgomery, C., Geske, R. & Bradley, A. Targeted disruption of the *c-src* proto-oncogene leads to osteopetrosis in mice. *Cell* **64**, 693–702 (1991).
64. Boyce, B. F., Yoneda, T., Lowe, C., Soriano, P. & Mundy, G. R. Requirement of *pp60c-src* expression for osteoclasts to form ruffled borders and resorb bone in mice. *J. Clin. Invest.* **90**, 1622–1627 (1992).
65. Schwartzberg, P. L. *et al.* Rescue of osteoclast function by transgenic expression of kinase-deficient Src in $src^{-/-}$ mutant mice. *Genes Dev.* **11**, 2835–2844 (1997).
66. Duong, L. T. *et al.* PYK2 in osteoclasts is an adhesion kinase, localized in the sealing zone, activated by ligation of α(v)β3 integrin, and phosphorylated by src kinase. *J. Clin. Invest.* **102**, 881–892 (1998).
67. Mattsson, J. P. *et al.* Isolation and reconstitution of a vacuolar-type proton pump of osteoclast membranes. *J. Biol. Chem.* **269**, 24979–24982 (1994).
68. Teitelbaum, S. L. Bone resorption by osteoclasts. *Science* **289**, 1504–1508 (2000).
69. Sly, W. S., Hewett-Emmett, D., Whyte, M. P., Yu, Y.-S. & Tashian, R. E. Carbonic anhydrase II deficiency identified as the primary defect in the autosomal recessive syndrome of osteopetrosis with renal tubular acidosis and cerebral calcification. *Proc. Natl Acad. Sci. USA* **80**, 2752–2756 (1983).
70. Kornak, U. *et al.* Mutations in the a3 subunit of the vacuolar H^+-ATPase cause infantile malignant osteopetrosis. *Hum. Mol. Genet.* **9**, 2059–2063 (2000).
71. Michigami, T. *et al.* Novel mutations in the a3 subunit of vacuolar H^+-adenosine triphosphatase in a Japanese patient with infantile malignant osteopetrosis. *Bone* **30**, 436–439 (2002).
72. Frattini, A. *et al.* Defects in TCIRG1 subunit of the vacuolar proton pump are responsible for a subset of human autosomal recessive osteopetrosis. *Nature Genet.* **25**, 343–346 (2000).
Although the molecular pathogenesis of most cases of human osteopetrosis remains enigmatic, this paper documents that many patients with the autosomal recessive form of the disease have mutations in the 116-kDa subunit of the osteoclast vacuolar H^+ATPase.
73. Scimeca, J.-C. *et al.* The gene encoding the mouse homologue of the human osteoclast-specific 116-kDa V-ATPase subunit bears a deletion in osteosclerotic (*oc/oc*) mutants. *Bone* **26**, 207–213 (2000).
74. Li, Y. P., Chen, W., Liang, Y., Li, E. & Stashenko, P. Atp6i-deficient mice exhibit severe osteopetrosis due to loss of osteoclast-mediated extracellular acidification. *Nature Genet.* **23**, 447–451 (2000).
75. Abu-Amer, Y., Ross, F. P., Schlesinger, P., Tondravi, M. M. & Teitelbaum, S. L. Substrate recognition by osteoclast precursors induces s-src/microtubule association. *J. Cell Biol.* **137**, 247–258 (1997).
76. Schlesinger, P. H., Blair, H. C., Teitelbaum, S. L. & Edwards, J. C. Characterization of the osteoclast ruffled border chloride channel and its role in bone resorption. *J. Biol. Chem.* **272**, 18636–18643 (1997).
77. Kornak, U. *et al.* Loss of the ClC-7 chloride channel leads to osteopetrosis in mice and man. *Cell* **104**, 205–215 (2001).
The authors establish that mice and humans that lack a chloride channel in the osteoclast ruffled membrane fail to acidify the resorptive microenvironment, and so develop osteopetrosis.
78. Gelb, B. D., Shi, G. P., Chapman, H. A. & Desnick, R. J. Pycnodysostosis, a lysosomal disease caused by cathepsin-K deficiency. *Science* **273**, 1236–1238 (1996).
79. Saftig, P. *et al.* Impaired osteoclastic bone resorption leads to osteopetrosis in cathepsin-K-deficient mice. *Proc. Natl Acad. Sci. USA* **95**, 13453–13458 (1998).
Consistent with the fact that cathepsin K deficiency is responsible for the bone-sclerosing disorder pycnodysostosis, the authors find mice that lack the enzyme have osteopetrosis owing to the failure of osteoclasts to degrade the collagenous component of bone.
80. Nishi, Y. *et al.* Determination of bone markers in pycnodysostosis: effects of cathepsin-K deficiency on bone matrix degradation. *J. Bone Miner. Res.* **14**, 1902–1908 (1999).
81. Motyckova, G. *et al.* Linking osteopetrosis and pycnodysostosis: regulation of cathepsin-K expression by the microphthalmia transcription factor family. *Proc. Natl Acad. Sci. USA* **98**, 5798–5803 (2001).
82. Dodds, R. A. *et al.* Human osteoclast cathepsin-K is processed intracellularly prior to attachment and bone resorption. *J. Bone Miner. Res.* **16**, 478–486 (2001).
83. Garnero, P. *et al.* The collagenolytic activity of cathepsin-K is unique among mammalian proteinases. *J. Biol. Chem.* **273**, 32347–32352 (1998).
84. Zaidi, M., Troen, B., Moonga, B. S. & Abe, E. cathepsin-K, osteoclastic resorption, and osteoporosis therapy. *J. Bone Miner. Res.* **16**, 1747–1749 (2001).
85. Lark, M. W. *et al.* A potent small molecule, nonpeptide inhibitor of cathepsin-K (SB 331750) prevents bone matrix resorption in the ovariectomized rat. *Bone* **30**, 746–753 (2002).
86. Anderson, D. M. *et al.* A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function. *Nature* **390**, 175–179 (1997).
87. Hughes, A. E. *et al.* Mutations in *TNFRSF11A*, affecting the signal peptide of RANK, cause familial expansile osteolysis. *Nature Genet.* **24**, 45–48 (2000).
This report provided the first evidence for mutations in RANK and documented their capacity to lead to enhanced osteoclastogenesis by autonomous activation of signals downstream of RANK.
88. Haslam, S. I. *et al.* Paget's disease of bone: evidence for a susceptibility locus on chromosome 18q and for genetic heterogeneity. *J. Bone Miner. Res.* **13**, 911–917 (1998).
89. Whyte, M. P. & Hughes, A. E. Expansile skeletal hyperphosphatasia is caused by a 15-base pair tandem duplication in *TNFRSF11A* encoding RANK and is allelic to familial expansile osteolysis. *J. Bone Miner. Res.* **17**, 26–29 (2002).
90. Whyte, M. P. *et al.* Osteoprotegerin deficiency and juvenile Paget's disease. *N. Engl. J. Med.* **347**, 175–184 (2002).
The authors elegantly show that deletion of the gene for OPG in humans, which is the decoy receptor for RANKL, results in greatly increased activation of RANK and hence bone resorption.
91. Hofbauer, L. C. & Schoppet, M. Osteoprotegerin deficiency and juvenile Paget's disease. *N. Engl. J. Med.* **347**, 1622–1623 (2002).
92. Cundy, T. *et al.* A mutation in the gene *TNFRSF11B* encoding osteoprotegerin causes an idiopathic hyperphosphatasia phenotype. *Hum. Mol. Genet.* **11**, 2119–2127 (2002).
93. Laurin, N., Brown, J. P., Morissette, J. & Raymond, V. Recurrent mutation of the gene encoding sequestosome 1 (SQSTM1/p62) in Paget disease of bone. *Am. J. Hum. Genet.* **70**, 1582–1588 (2002).
94. Hocking, L. J. *et al.* Domain-specific mutations in sequestosome 1 (*SQSTM1*) cause familial and sporadic Paget's disease. *Hum. Mol. Genet.* **11**, 2735–2739 (2002).

An important study showing that Paget disease of bone can arise as a result of mutations of an adaptor protein that couples RANK to more distal signalling events.
95. Geetha, T. & Wooten, M. W. Association of the atypical protein kinase C-interacting protein p62/ZIP with nerve growth factor receptor TrkA regulates receptor trafficking and Erk5 signaling. *J. Biol. Chem.* **278**, 4730–4739 (2003).
96. Moscat, J. & Diaz-Meco, M. T. The atypical protein kinase Cs: functional specificity mediated by specific protein adapters. *EMBO Rep.* **1**, 399–403 (2000).
97. Rohrschneider, L. R., Fuller, J. F., Wolf, I., Liu, Y. & Lucas, D. M. Structure, function, and biology of SHIP proteins. *Genes Dev.* **14**, 505–520 (2000).
98. March, M. E. & Ravichandran, K. Regulation of the immune response to SHIP. *Semin. Immunol.* **14**, 37–47 (2002).
99. Helgason, C. D. *et al.* Targeted disruption of SHIP leads to hemopoietic perturbations, lung pathology, and a shortened life span. *Genes Dev.* **12**, 1610–1620 (1998).
100. Takeshita, S. *et al.* SHIP-deficient mice are severely osteoporotic due to increased numbers of hyper-resorptive osteoclasts. *Nature Med.* **8**, 943–949 (2002).
The first genetic evidence that derangements in phosphoinositide metabolism can lead to greater bone loss as a result of increased osteoclast number and activity.
101. Ware, M. D. *et al.* Cloning and characterization of human SHIP, the 145-kD inositol 5- phosphatase that associates with SHC after cytokine stimulation. *Blood* **88**, 2833–2840 (1996).
102. Khosla, S., Melton, L. J. & Riggs, B. L. Estrogen and the male skeleton. *J. Clin. Endocrinol. Metab.* **87**, 1443–1450 (2002).
103. Pacifici, R. *et al.* Ovarian steroid treatment blocks a postmenopausal increase in blood monocyte interleukin 1 release. *Proc. Natl Acad. Sci. USA* **86**, 2398–2402 (1989).
104. Pacifici, R. *et al.* Effect of surgical menopause and estrogen replacement on cytokine release from human blood mononuclear cells. *Proc. Natl Acad. Sci. USA* **88**, 5134–5138 (1991).
105. Lorenzo, J. A. *et al.* Mice lacking the type I interleukin-1 receptor do not lose bone mass after ovariectomy. *Endocrinology* **139**, 3022–3025 (1998).
106. Ammann, P. *et al.* Transgenic mice expressing soluble tumor necrosis factor-receptor are protected against bone loss caused by estrogen deficiency. *J. Clin. Invest.* **99**, 1699–1703 (1997).
107. Roggia, C. *et al.* Up-regulation of TNF-producing T cells in the bone marrow: a key mechanism by which estrogen deficiency induces bone loss *in vivo*. *Proc. Natl Acad. Sci. USA* **98**, 13960–13965 (2001).
108. Kimble, R. B. *et al.* Estrogen deficiency increases the ability of stromal cells to support murine osteoclastogenesis via an interleukin-1 and tumor necrosis factor-mediated stimulation of macrophage colony-stimulating factor production. *J. Biol. Chem.* **271**, 28890–28897 (1996).
109. Cenci, S., Weitzmann, M. N., Gentile, M. A., Aisa, M. C. & Pacifici, R. M-CSF neutralization and Egr-1 deficiency prevent ovariectomy-induced bone loss. *J. Clin. Invest.* **105**, 1279–1287 (2000).
110. Cenci, S. *et al.* Estrogen deficiency induces bone loss by enhancing T cell production of TNFα. *J. Clin. Invest.* **106**, 1229–1237 (2000).
111. Lam, J. *et al.* TNF-α induces osteoclastogenesis by direct stimulation of macrophages exposed to permissive levels of RANK ligand. *J. Clin. Invest.* **106**, 1481–1488 (2000).
112. Cenci, G. *et al.* Estrogen deficiency causes bone loss by upregulating T cell proliferation and lifespan through IFNγ-induced class II transactivator. *Proc. Natl Acad. Sci. USA* (in the press).
113. Eghbali-Fatourechi, G. *et al.* Role of RANK ligand in mediating increased bone resorption in early postmenopausal women. *J. Clin. Invest.* **111**, 1221–1230 (2003).
114. Takayanagi, H. *et al.* T-cell-mediated regulation of osteoclastogenesis by signalling cross-talk between RANKL and IFN-γ. *Nature* **408**, 600–605 (2000).
115. Takayanagi, H. *et al.* RANKL maintains bone homeostasis through c-Fos-dependent induction of interferon-β. *Nature* **416**, 744–749 (2002).
116. Passeri, G., Girasole, G., Jilka, R. L. & Manolagas, S. C. Increased interleukin-6 production by murine bone marrow and bone cells after estrogen withdrawal. *Endocrinology* **133**, 822–828 (1993).
117. Jilka, R. L. *et al.* Increased osteoclast development after estrogen loss: mediation by interleukin-6. *Science* **257**, 88–91 (1992).
118. Kawasaki, K. *et al.* Osteoclasts are present in gp130-deficient mice. *Endocrinology* **138**, 4959–4965 (1997).
119. Choi, S. J. *et al.* Antisense inhibition of macrophage inflammatory protein 1-α blocks bone destruction in a model of myeloma bone disease. *J. Clin. Invest.* **108**, 1833–1841 (2001).
120. Horwood, N. J. *et al.* Interleukin 18 inhibits osteoclast formation via T cell production of granulocyte macrophage colony-stimulating factor. *J. Clin. Invest.* **101**, 595–603 (1998).
121. Wei, S., Wang, M. W., Teitelbaum, S. L. & Ross, F. P. Interleukin-4 reversibly inhibits osteoclastogenesis via inhibition of NF-κB and MAP kinase signaling. *J. Biol. Chem.* **21**, 6622–6630 (2001).
122. Udagawa, N. *et al.* Interleukin-18 (interferon-γ-inducing factor) is produced by osteoblasts and acts via granulocyte/macrophage colony-stimulating factor and not via interferon-γ to inhibit osteoclast formation. *J. Exp. Med.* **185**, 1005–1012 (1997).
123. Horwood, N. J., Elliott, J., Martin, T. J. & Gillespie, M. T. IL-12 alone and in synergy with IL-18 inhibits osteoclast formation *in vitro*. *J. Immunol.* **166**, 4915–4921 (2001).
124. Faccio, R., Novack, D. V., Zallone, A., Ross, F. P. & Teitelbaum, S. L. Dynamic changes in the osteoclast cytoskeleton in response to growth factors and cell attachment are controlled by b3 integrin. *J. Cell Biol.* (in the press).
125. Kong, Y. Y. *et al.* OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. *Nature* **397**, 315–323 (1999).
126 Ross, F. P. Interleukin 7 and estrogen-induced bone loss. *Trends Endocrinol. Metab.* **14**, 147–149 (2003).

Acknowledgements
We thank M. Whyte for assistance with the gain of function section. The authors are recipients of National Institutes of Health support.

Online Links

DATABASES
The following terms in this article are linked online to:
LocusLink: http://www.ncbi.nlm.nih.gov/LocusLink
Bcl2 | *Ctsk* | *Fos* | *Fosl1* | *Ifnar1* | *INPP5D* | *SPI1* | *SQSTM1* | *Tcirg1* | *Tnf* | *Tnfrsf11a* | *TNFRSF11A* | *Tnfrsf11b* | *TNFRSF11B* | *Tnfsf11* | *Traf6*
OMIM: http://www.ncbi.nlm.nih.gov/omim
Glanzmann thrombasthenia | hereditary expansile polyostotic osteolytic dysplasia | osteopetrosis | osteoporosis | Paget disease of bone | PDB1 | PDB4 | pycnodysostosis
Access to this interactive links box is free online.

JBC Papers in Press. Published on May 3, 2010 as Manuscript M109.091124
The latest version is at http://www.jbc.org/cgi/doi/10.1074/jbc.M109.091124

3BP2 ADAPTER PROTEIN IS REQUIRED FOR RANKL-INDUCED OSTEOCLAST DIFFERENTIATION OF RAW264.7 CELLS

Amel GuezGuez[1,2], Virginie Prod'homme[1,2], Xavier Mouska[1], Alice Baudot[1,2], Claudine Blin-Wakkach[2,3], Robert Rottapel[4], and Marcel Deckert[1,2]

From INSERM, UMR576, Hôpital de l'Archet, Nice, F-06202 France[1], University of Nice Sophia-Antipolis, Nice, F-06103 France[2], CNRS, FRE2943, Faculté de Médecine, Nice, F-06107 France[3], and Ontario Cancer Institute, Toronto Medical Discovery Tower, Toronto, Canada[4].

Running Title: 3BP2 and osteoclast differentiation

Address correspondence to: Marcel Deckert, INSERM UMR576, Hôpital de l'Archet 1, Route Saint-Antoine de Ginestière, F-06202, Nice Cedex 3, France. Phone: 33-492-157-700. Fax: 33-492-157-709. E-mail: deckert@unice.fr

The adapter protein 3BP2 (also known as SH3BP2, Abl SH3-binding protein 2) has been involved in leukocyte signaling and activation downstream Immunoreceptors. Genetic studies have further associated 3BP2 mutations to the human disease Cherubism, and inflammation and bone dysfunction in mouse. However, how wild-type 3BP2 functions in macrophage differentiation remains poorly understood. In this study, using siRNA-mediated silencing of 3BP2 in the RAW264.7 monocytic cell line, we show that 3BP2 was required for RANKL-induced differentiation of RAW264.7 cells into multinucleated mature osteoclasts, but not for GM-CSF/IL4-induced differentiation into dendritic cells. 3BP2 silencing was associated with impaired activation of multiple signaling events downstream of RANK, including actin reorganization, Src, ERK and JNK phosphorylation, and up-regulation of osteoclastogenic factors. In addition, 3BP2-knockdown cells induced to osteoclast by RANKL displayed reduced increase of Src and NFATc1 mRNA and protein expression. Importantly, 3BP2 interacted with Src, Syk, Vav, and Cbl in monocytic cells and the introduction of constitutively active mutants of Src and NFATc1 in 3BP2 deficient cells restored osteoclast differentiation. Finally, the expression of a 3BP2 cherubism mutant was found to promote increased Src activity and NFAT-dependent osteoclast formation. Together, this study demonstrates that wild type 3BP2 is a key regulator of RANK-mediated macrophage differentiation into osteoclast through Src and NFATc1 activation.

Osteoclasts are multinucleated bone-resorbing cells, differentiating from CD11b+ hematopoietic cells of the monocyte/macrophage lineage. The interaction between bone marrow stroma, osteoblasts, hematopoietic cells, and the immune system is determinant for bone homeostasis and osteoclastogenesis (1,2). Osteoclast differentiation is essentially triggered by two hematopoietic factors, the member of the TNF superfamily of cytokine receptor activator of NFκB (RANK) ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) (1). In response to osteotropic factors, osteoblasts express RANKL, which binds RANK on osteoclast progenitors cell surface leading to osteoclast maturation. Consistently, mice lacking RANKL or RANK exhibit an osteopetrotic phenotype, associated with defects in bone resorption and a lack of osteoclasts (3). Stimulation of mononuclear osteoclast precursors by RANKL and M-CSF regulates growth, differentiation, fusion and survival, leading to functional multinucleated osteoclasts (1). Mature osteoclasts express typical markers including tartrate-resistant acidic phosphatase (TRAP) and calcitonin receptor, and adhere to the bone surface through a ring of polymerized actin, adhesion receptors ($\alpha v\beta 3$) and cytoskeleton molecules, forming a sealed resorbing compartment, in which acidification and secretion of proteolytic enzymes allow the resorption of the bone matrix (1,4).

Osteoclast stimulation by RANKL and M-CSF triggers the activation of intracellular events involving a large number of signaling molecules, including the adapters TRAF6 (5), Dap12 and FcRγ (6,7), the Rho GTPases activator Vav3 (8), phosphatidylinositol 3-kinase

(PI3K) (9), and MAP kinases family members ERK, JNK, and p38. The Src (10), Syk (7,11) and Tec (12) protein tyrosine kinases (PTKs) families are other key molecules in osteoclastogenesis downstream RANK, ITAM-bearing molecules, integrins and c-fms, the M-CSF receptor (1,12,13). These signal transduction pathways ultimately converge to the activation of several transcription factors such as NFκB, c-fos, PU.1, and microphathalmia-associated transcription factor (MITF) (4). Another crucial transcriptional event during osteoclastogenesis is the up-regulation of NFATc1 downstream RANK and intracellular calcium (Ca2+) signaling (14,15). NFATc1 is a member of the NFAT family of transcription factors which transcriptional activities are regulated by the calcium (Ca2+)-dependent serine/threonine phosphatase calcineurin (16). Inhibiting calcineurin activity by cyclosporine A and FK506 suppressed osteoclast differentiation *in vitro* (17). Conversely, the expression of constitutively activated NFATc1 promotes osteoclast differentiation in the absence of RANKL (14,18). Therefore, NFATc1 appears to be necessary and sufficient for osteoclastogenesis.

Adapter proteins are key components of leukocyte Immunoreceptor signal transduction pathways coupled to PTKs (19,20). Molecular scaffolds composed of adapter proteins and enzymes are assembled and activated at the plasma membrane by Src and/or Syk PTKs. These scaffolds transduce signals to the cytoplasm, cytoskeleton and nucleus to activate lipid and calcium signaling, gene expression and metabolic changes involved in leukocyte proliferation, differentiation and motility. Others and we have identified a regulatory role of 3BP2 (or SH3BP2) in Immunoreceptor signaling, including T (21,22), B (23-26), NK (27), and mast (28) cells. Importantly, 3BP2 associates several signaling proteins such as Src/Syk kinases, Vav proteins, and PLCγ, involved in NFAT activation (reviewed in (29,30)). Consistently, 3BP2 was found to positively regulate the activity of NFAT in T and B cells (21,23). In addition, studies in mouse deficient for 3BP2 expression have shown that 3BP2 regulates B cell development and BCR-mediated B cell activation and calcium mobilization (24,25). Finally, genetic evidence linking 3BP2 to the human genetic bone disease Cherubism (31,32) indicates that 3BP2 also plays a crucial role during inflammation and bone remodeling. Cherubism is an autosomal dominant disorder characterized by erosion of maxillar and mandibular bone with resultant dental and facial deformity due to excessive osteoclast activity and giant cell granuloma formation (33). The signaling alterations of a mutant form of 3BP2 as observed in a mouse model of Cherubism include increased TNFα production by hyperactive macrophages, associated with systemic inflammation, aberrant osteoclast activities and osteoporosis (32). Interestingly, genetic inactivation of NFATc1 in Cherubism mice prevented bone loss (34), suggesting that NFAT activation by 3BP2 is a critical step during osteoclastogenesis. However, how wild type 3BP2 exactly functions in RANK-mediated osteoclast differentiation has not yet been elucidated.

In this study, we have investigated the role of 3BP2 during osteoclast differentiation and RANK signaling. Using RNAi blocking experiments in the RAW264.7 monocyte/macrophage cell line, we show that the absence of 3BP2 in pre-osteoclasts is associated with a severe reduction of osteoclast formation and increased expression of osteoclastogenic factors. The absence of 3BP2 resulted in decreased RANK-mediated actin cytoskeleton remodeling, Src phosphorylation, and activation of multiple signaling pathways involved in RANK signaling, as well as a deregulated expression of NFATc1. 3BP2 interacted with signaling proteins, including Src, Syk, Vav1 and Cbl in resting cells and the introduction of constitutively active mutants of Src and NFATc1 in 3BP2 deficient cells restored osteoclast differentiation. In addition, the expression of a 3BP2 cherubism mutant was found to promote increased Src activity and NFAT-dependent osteoclast formation. Together, this study demonstrates that wild type 3BP2 is a key regulator of RANK-mediated osteoclastogenesis through Src and NFATc1 activation.

Experimental Procedures

Cell line and culture- RAW264.7 cells were purchased from American Type Culture Collection (Manassas, VA). Cells were maintained at 37°C, 5% CO2 in α-MEM (minimum Eagle's medium) (Lonza,

Walkersville, MD) supplemented with 10% fetal bovine serum (Hyclone, USA), penicillin and streptomycin (Gibco BRL, UK). For stimulation, cells were deprived from serum for 12 hours in α-MEM. 5.10^6 cells/ml were then stimulated at 37°C for the indicated time with or without the indicated cytokine.

Reagents, Antibodies and Plasmids - All chemicals were from Sigma Aldrich (St Louis, MO), except the pharmacological inhibitors PP2 and cyclosporine, which were from Merck (Darmstadt, Germany). Soluble RANKL was from R&D Systems (Minneapolis, MN), and M-CSF, GM-CSF, and IL-4 from ImmunoTools (Germany). Anti-3BP2 antibodies were described before (23). Antibodies against phosphorylated forms of Src, Syk, ERK1/2, p38, JNK, MEK1/2, Akt, IKKα/β, total Src, total Akt, and HRP-conjugated secondary antibodies were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against ERK2 and 0NFATc1 were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-V5 mAb was from Invitrogen (The Netherlands) and anti-hemagglutinin (HA) from Roche (Switzerland). The wild type and R415P 3BP2 cDNAs bearing a N-terminal V5 epitope tag (23) was cloned into the murine leukemia virus-based LZRS-IRES-GFP retroviral vector. The plasmids encoding the VIVIT peptide, Src Y527F, and constitutively active NFATc1 (plasmids 11106, 13660, 11102, respectively) were from Addgene (Cambridge, MA).

Generation of 3BP2-knockdown RAW264.7 cells - three short hairpins RNA (shRNA) expressing plasmids were generated using the Block-iT RNAi vector kit (Invitrogen). The complementary nucleotide sequences targeting position 381-401, 488-508 and 507-527 of mouse 3BP2 RNA were the following: 3BP2 381 sense, 5'-CACCGGAAATTGGCCACTTCCATGA*CGAA*TCATGGAAGTGGCCAATTTCCTTTT-3'; 3BP2 381 antisense, 5'-AAAAGGAAATTGGCCACTTCCATGA*TTC*GTCATGGAAGTGGACCATTTCC-3', 3BP2 488 sense, 5'-CACCGCCTGTCTTCATATCCCATGG*CGAA*CCATGGGATATGAAGACAGGCTTTT-3'; 3BP2 488 antisense 5'-AAAAGCCTGTCTTCATATCCCATGG*TTCG*CCATGGGATATGAAGACAGGC-3', 3BP2 507 sense, 5'-CACCGGACAATGAAGATTACGAACA*CGA*A TGTTCGTAATCTTCATTGTCCTTTT-3'; 3BP2 507 antisense, 5'- AAAA GGACAATGAAGATTACGAACA *TTCG* TCTTCGTAATCTTCATTGTCC-3'. Following annealing and ligation into pENTR/H1/TO, the 3BP2 shRNA vectors were transfected into RAW264.7 cells using lipofectamine (Invitrogen). As a control, RAW264.7 cells were transfected with the same amount of a LacZ shRNA construct. Stably transfected cells were selected using 100 µg/ml of zeocin (Invitrogen), and stable suppression of 3BP2 expression was evaluated by immunoblotting.

In vitro differentiation of osteoclasts and dendritic cells - For osteoclastogenesis assays using the RAW264.7 cell line, cells were plated in a 12-well plate at a cell density of 10,000 cells/well in the presence of 40 ng/ml RANKL, combined or not with 30 ng/ml M-CSF, and cultured for 4-5 days. Fresh culture medium containing RANKL was added on day 3. Cells were stained for TRAP activity with the leukocyte acid phosphatase kit (Sigma-Aldrich). Multinucleated TRAP-positive cells were counted and scored by microscopy. To determine complexity and size differences, OC were counted by number of nuclei and size. TRAP-positive cells with more than three nuclei and larger than 100µm in diameter were counted as osteoclasts.

For dendritic cell differentiation, RAW264.7 cells were cultured with recombinant granulocyte-macrophage colony stimulating factor (GM-CSF, 100 ng/ml) and IL-4 (10 ng/ml) for 6 days. For maturation of dendritic cells, lipopolysaccharide (LPS; 10 µg/ml) was added for 2 additional days. After 8 days, cells were analyzed for their morphology under a Zeiss Axiovert 40C light microscope, or stained with FITC-, or phycoerythrin-conjugated antibodies reactive to CD11c, CD80 or CD86 (all purchased form Becton-Dickinson), followed by flow cytometry analysis on a FACScan (Becton Dickinson).

F-actin fluorescence staining of osteoclast - Cells were seeded onto sterile chamber slide and treated with RANKL (40 ng/ml) for the indicated times. After washing, cells were fixed with 3.7% formaldehyde in PBS, permeabilized with 0.2% Triton X-100 for 10 min, washed twice in PBS containing 1% BSA and incubated

with 100 ng/ml of TexasRed phalloidin (Invitrogen) at 22°C in a humidified atmosphere for 30 min. After staining, cells were washed with PBS, rinsed with water and mounted with Fluoromount (Sigma-Aldrich). Polymerized actin was visualized using a Zeiss Axiovert 200M fluorescence microscope (Zeiss Leica Microsystems, ON) equipped by a Hamamatsu ORCA-ER digital camera (Hamamatsu, Japan). Image analysis was performed with Volocity software (Improvision Inc, Waltham, MA). Quantification of F-actin content in osteoclasts was performed by flow cytometry. Cells were scraped off culture dishes, fixed with 3.7% formaldehyde in PBS, and permeabilized with 0.05% saponin in PBS, 1% BSA for 20 min at room temperature. Cells were washed and labeled with 100 ng/ml AlexaFluor 488-conjugated phalloidin (Invitrogen) for 30 min at 22°C. Following three washes with cold PBS, 1% BSA, polymerized actin was measured by flow cytometry. Data are expressed as mean channel fluorescence intensity for each sample.

Immunoprecipitation and Immunoblotting –

Cells were lysed at $1x10^8$ cells/ml in ice-cold lysis buffer (1% Triton in 150 mM NaCl, 50 mM Tris-HCl [pH 7.5], 0.1% SDS, 0.1% sodium deoxycholate, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM PMSF) for 30 min on ice. Cleared lysates were then incubated for 3 h at 4°C with the indicated antibodies and 1 h with protein G-Sepharose beads (Sigma). Pellets were then washed three times with ice-cold lysis buffer and resuspended in SDS sample buffer. Eluted immunoprecipitates or whole cell lysates were separated by SDS-PAGE, and analyzed by immunoblotting as described before (23).

Transfection, Luciferase Reporter, and Complementation assays - The reporter plasmid pTRAP-luc was a kind gift from H. Takayanagi (Tokyo Medical and Dental University, Japan) (14). The luciferase reporter constructs for NFAT and AP-1 activities, and the 3BP2 expression constructs were described before (21,23). Transient transfections into RAW264.7 cells were performed using the AMAXA nucleofection system (Lonza, Germany). Briefly, 2.10^6 cells were washed and resuspended in 100 µl of Buffer V, mixed with 5µg of reporter plasmid along with the indicated expression plasmids and a Renilla luciferase plasmid (Promega), and electroporated using program D-032 on the AMAXA nucleofection device. Following incubation at 37°C for 16 hours, cells were stimulated or not with RANKL (40-100 ng/ml) for 24-48h at 37°C. Dual luciferase reporter assays were performed as described (23). Normalized luciferase activities were determined in triplicate and expressed as fold increase relative to basal activities measured in control vector-transfected cells.

For complementation assays, cells were transfected with mock retroviral expression vectors, constitutively active NFATc1 expression vector, or a combination of the Src Y527F expression vector and EGFP expression vector, as described above. After 48 hours of transfection, the GFP-positive transfected cells were purified on a FACSAria cell sorter (Beckton-Dickinson) and subjected to *in vitro* differentiation assays and immunoblotting analysis.

Real-time quantitative PCR - Total RNAs from RAW264.7 cells were reverse transcribed using the High Capacity cDNA Archive random priming Kit (Applied Biosystems). Real-time quantitative PCR (qRT-PCR) was performed using a 7900HT Sequence Detector System (Applied Biosystems) and the SYBR Green dye detection protocol as previously described (35). Relative expression level of target genes mRNA between control (X) and sample (Y) was calculated using the formula ΔC_T Y – DC_T X and expressed as fold over control ($2\Delta\Delta C_T$).

Statistical Analysis - Data are expressed as the mean ± SD. The statistical significance of differences between the experimental groups was determined by an unpaired Student's *t* test. Effects with a *p* value less than 0.05 were considered statistically significant.

RESULTS

Suppression of osteoclast differentiation in 3BP2-knockdown RAW264.7 cells. To examine the involvement of 3BP2 in the regulation of osteoclast differentiation, we generated stable and inducible 3BP2-knockdown cell models. Three short hairpin RNA (shRNA) expressing plasmids targeting three different sequences of 3BP2 were generated and were stably transfected into the RAW264.7 cell line. As shown in Figure 1A and 1B, two shRNA constructs designated as sh3BP2 (381) and sh3BP2 (507) completely suppressed the

expression of 3BP2 and RANKL-induced formation of TRAP-positive multinucleated cells. In contrast, the shRNA expressing plasmid sh3BP2 (488), which had no effect on 3BP2 expression as compared to the shLacZ control vector, did not interfere with RANKL-induced cell-cell fusion. Quantitative analysis showed that the lack of 3BP2 dramatically reduced the number of TRAP-positive multinuclear osteoclasts, and the number of nuclei per TRAP-positive cells, induced upon RANKL stimulation (Figure 1B). We determined that neither spontaneous proliferation of sh3BP2 (381) cells was significantly modified compared to shLacZ cells (Supplementary Figure S1), nor RANK expression was different on both cell types (not shown). We also controlled that the combined stimulation of the sh3BP2 (381) RAW264.7 cell clone by RANKL in the presence of M-CSF had no effect on the impaired differentiation of 3BP2-silenced cells (Supplementary Figure S2). To further document the role of 3BP2 in osteoclast differentiation, we selected the sh3BP2 (381) RAW264.7 cell clone (referred to as sh3BP2 cells for simplification) to examine the expression of osteoclastogenic factors using qRT-PCR analysis and luciferase reporter assays. First, RAW264.7 cells were transiently transfected with a TRAP promoter-luciferase plasmid and the luciferase activity was measured. As shown in Figure 1C, 3BP2-knockdown cells (sh3BP2) showed a marked reduction of both basal and RANKL-induced TRAP promoter activities as compared to control cells (shLacZ). Using qRT-PCR analysis, we next examined the expression of osteoclastogenic factors in control or 3BP2-knockdown cells treated with sRANKL for different time. We found that sRANKL-induced expression of TRAP, calcitonin receptor, and cathepsin K was severely impaired in 3BP2 deficient cells (Figure 1D). Thus, expression of 3BP2 is required for RANKL-induced differentiation of RAW264.7 cell into multinucleated TRAP-positive osteoclasts.

3BP2 is not required for dendritic cell differentiation. It is known that monocytes/macrophages exposed to GM-CSF/IL-4 can acquire DC phenotype and function (36). To examine whether 3BP2 is involved in DCs differentiation, sh3BP2 RAW264.7 cells were cultured with GM-CSF and IL-4 for 6 days. Maturation of dendritic-like cells was further induced by treatment with LPS for 2 additional days. Cell differentiation was then analyzed by phase contrast microscopy and flow cytometry. After 6 days of treatment with GM-CSF/IL-4, a morphological transformation from macrophage-like into dendritic-like cells was observed for both control and 3BP2-knockdown cells, a process further increased following incubation with LPS for another 2 days (Figure 2A). Dendritic morphology was characterized by an increase in cell size and multiple membrane protrusions. Dendritic-like phenotypic change in either control or 3BP2 deficient cells was also evidenced by the increased membrane expression of typical dendritic cell markers such as CD11c, CD80 (B7.1) and CD86 (B7.2) following GM-CSF/IL-4 + LPS treatment (Figure 2B). Finally, the lack of 3BP2 expression in RAW264.7 cells had no significant effects on the early biochemical events triggered by cell stimulation with GM-CSF/IL-4, including the activation of ERK, p38, Akt and IKKα/β phosphorylation (Figure 2C).

3BP2 is required for organization of the osteoclast actin cytoskeleton. Actin cytoskeleton remodeling plays an essential role during osteoclast differentiation and function (1). To examine the implication of 3BP2 in the osteoclast actin reorganization, sh3BP2 RAW264.7 cells and shLacZ control cells were cultured for 4 days with sRANKL and subjected to immunofluorescence analysis with fluorescent phalloidin to detect actin organization. After 2 days of stimulation, control cells showed increased actin polymerization associated with cell spreading, whereas those lacking 3BP2 exhibited altered F-actin content and spreading. The architectural abnormalities of 3BP2-knockdown osteoclasts were evident after 3 days of RANKL treatment. Whereas control cells showed well-formed actin rings and cell fusion at days 3 and 4, no expression of 3BP2 in osteoclasts led to a dramatic reduction of actin polymerization associated with cell-cell fusion (Figure 3A). By using flow cytometry, we quantified how 3BP2 interferes with actin polymerization in response to RANKL during osteoclast differentiation. Control cells showed a 2 to 3-fold increase in F-actin content after one day of RANKL stimulation and the total amount of polymerized actin remained stable during the next 3 days of treatment. In contrast, 3BP2-knockdown cells exhibited only a 2-fold increase in F-actin between day 1 and 2 of culture with

RANKL, followed by a dramatic fall of actin polymerization associated with impaired cell-cell fusion (Figure 3B). Functional osteoclasts adhere to the bone surface through adhesion receptors, including $\alpha v\beta 3$ integrin and CD44, connected to the actin cytoskeleton network. Lack or failure to up-regulate their expression during osteoclast maturation results in severe cytoskeletal defects and abnormal bone resorption (37,38). We therefore examined the integrity of integrin $\alpha v\beta 3$ and CD44 signaling pathways in the absence of 3BP2. As expected, control osteoclasts treated with RANKL for 2 to 4 days strongly up-regulated the mRNAs encoding αv and $\beta 3$ integrin subunits, and CD44. In contrast, no significant increase of $\alpha v\beta 3$ and CD44 expression was detected in 3BP2-knockdown osteoclasts (Figure 3C). Consistently, RANKL-induced phosphorylation of FAK and ERM, two cytoplasmic regulators of the $\alpha v\beta 3$ and CD44 signaling pathways, respectively, was severely altered in sh3BP2 cells as compared to control cells (Figure 3D).

3BP2 regulates multiple signaling pathways in response to RANKL. Binding of RANKL to its receptor RANK triggers the recruitment and activation of multiple signaling effectors, leading to transcriptional regulation of osteoclastogenic factors (1). We therefore examined RANKL-induced early signaling pathways in 3BP2-knockdown macrophages. As shown in Figure 4A, RANKL-stimulated phosphorylation of Src, Akt and MAPKs ERK1/2 and JNK were dramatically affected in the absence of 3BP2. In contrast, the phosphorylation of p38 was not modified. Upon RANKL stimulation, 3BP2-knockdown cells also displayed reduced phosphorylation of MEK1, an upstream activator of ERK1/2, and of IKKα/β, an upstream activator of the transcription factor NFκB. These early signaling pathways increase the activity of multiple transcription factors playing essential roles during osteoclastogenesis. We therefore examined whether 3BP2 expression can regulate the transcriptional activities of AP-1, NFκB and NFAT using a luciferase reporter assay in RAW264.7 cells. A marked reduction of basal and RANKL-induced AP-1 and NFAT activities was observed in 3BP2-knockdown cells as compared to control cells (Figure 4B). In addition, we found reduced levels of the mRNA encoding the transcription factors c-Fos and NFATc1, and the kinase Src (Figure 4C), whereas the expression of NFATc2 and NFATc3, two transcription factors related to NFATc1, was not significantly modified in the absence of 3BP2 (Figure 4C). In addition, the mRNA expression of PU.1, a transcription factor regulating osteoclastogenesis, was unaffected in the absence of 3BP2 (data not shown). Consistent with the impaired increase of mRNA expression, 3BP2-knockdown cells induced to osteoclast by RANKL for 2 to 4 days displayed no up-regulation of NFATc1 and c-Src at protein levels, compared to control shLacZ cells (Figure 4D). Thus, 3BP2 appears to regulate multiple signaling pathways downstream RANKL-RANK interaction, including the major osteoclastogenic factor NFATc1.

Ectopic expression of active Src and NFAT proteins rescues osteoclast formation by 3BP2 knockdown macrophages. Previous studies have implicated 3BP2 in NFAT activation in leukocytes, and shown its interaction with members of the Src family of PTKs (29,30). As shown in Figure 5A, 3BP2 co-immunoprecipitated with c-Src in resting monocytic cells. Consistent with other studies in lymphocytes (21,23), 3BP2 also interacted with Syk, Vav1, and Cbl in RAW264.7 cells. We next determined the contribution of Src and NFATc1 to 3BP2-dependent osteoclast formation. To this end, we introduced in 3BP2-knockdown RAW264.7 cells either control retroviruses or constitutively active forms of c-Src (SrcY527F) (39) (Figure 5B) or NFATc1 (caNFATc1) (40) (Figure 6A), and examined osteoclast formation. Expression of SrcY527F in RANKL-stimulated 3BP2 knockdown macrophages partially restored cell-cell fusion and the formation of TRAP-positive multinucleated cells (Figure 5C). Osteoclast-like cell number (Figure 5D, left panel) and size (Figure 5D, right panel) were significantly increased in RANKL-stimulated sh3BP2 cells expressing SrcY527F, as compared to RANKL-stimulated sh3BP2 cells expressing a control construct. Importantly, as shown in Figure 5B, overexpression of active Src in control and 3BP2 knockdown cells induced the phosphorylation of Syk Tyr352 and ERK1/2, two events critically involved in several aspects of osteoclast formation and function (2). The constitutive phosphorylation of SrcY527F on

Tyr416 was confirmed by immunoblot analysis (Figure 5B).

Next, we assessed the differentiation of cells expressing a retroviral construct encoding a HA-tagged constitutively active form of NFATc1. Expression of caNFATc1 in 3BP2-knockdown cells was found to completely restore the formation of TRAP-positive, multinucleated cells in RANKL-treated cells (Figure 6B). In addition to morphology, the number (Figure 6C, left panel) and size (Figure 6C, right panel) of osteoclast-like cells formed by 3BP2-knockdown macrophages expressing caNFATc1 reached levels comparable to control conditions. The expression of HA-tagged caNFATc1 was confirmed by immunoblot analysis (Figure 6A). Together, these data suggest that 3BP2 acts upstream of Src and NFATc1 in RANK-induced signaling pathways of osteoclast formation.

Expression of 3BP2 cherubism mutant increases c-Src activation and osteoclast formation in RAW264.7 cells. Genetic studies have linked mutations of 3BP2 to the bone disease Cherubism in human and mice (31,32), and genetic studies have linked 3BP2 mutation to enhanced phosphorylation of Syk in myeloid cells (32). To assess whether 3BP2 cherubism mutant proteins also affect c-Src activity and osteoclast formation in our system, we used retroviral constructs encoding V5-tagged forms of wild-type 3BP2 and 3BP2 R415P cherubism mutant, which were expressed in RAW264.7 cells through IRES-GFP bicistronic retroviral constructs (Figure 7A). After 2 days, GFP+ cells were sorted by FACS and subjected to RANKL stimulation. As shown in Figure 7B, expression of the R415P cherubism mutant significantly increased the formation of TRAP+ multinucleated osteoclasts, compared to control and over expressed wild type 3BP2. Ectopic expression of 3BP2 R415P was also accompanied by a marked increase of Src phosphorylation on the activating site Tyr418 (Figure 7C). Consistent with studies by Aliprantis et al. (34), we also show that the increased osteoclast formation induced by the 3BP2 R415P mutant required NFAT activity as the observed effect was completely abrogated by the expression of the selective NFAT inhibitor VIVIT-GFP (41) (supplementary Figure S3). Together, these data indicate that 3BP2 mutant proteins promote increased Src activity and NFAT-dependent osteoclast formation in RAW264.7 cells.

DISCUSSION

Genetic evidence linking mutant 3BP2 proteins to the bone disease cherubism (31,32,34) indicates that 3BP2 may play a crucial role during inflammation and bone remodeling. However, little is known about the molecular mechanisms by which wild-type endogenous 3BP2 regulates osteoclastogenesis. Using RNAi blocking experiments in the RAW264.7 monocyte/macrophage cell line, we show here that absence of 3BP2 in pre-osteoclasts dramatically impaired their ability to form TRAP+ multinucleated cells following RANKL stimulation. The defects in osteoclastogenesis observed in the absence of 3BP2 resulted from a decreased RANK-mediated actin cytoskeleton remodeling and activation of multiple signaling pathways, including Src phosphorylation, and impaired NFATc1 expression. Genetic complementation of 3BP2 deficient cells with constitutively active mutants of Src and NFATc1 restored osteoclast differentiation. In addition, the expression of a 3BP2 cherubism mutant was found to promote increased Src activity and NFAT-dependent osteoclast formation. Our study demonstrates that wild type 3BP2 is required for RANKL-induced osteoclastogenesis through its regulation of signaling pathways involving Src and NFATc1 activation.

In leukocytes, including T and B lymphocytes, NK and mast cells, 3BP2 interacts with intracellular proteins such as Src and Syk PTKs, Vav proteins, and PLCγ, that have been involved in calcium signaling and NFAT activation downstream ITAM-containing Immunoreceptors and adaptors (29,30). Thus, our data indicate that osteoclasts constitute another type of leukocytes in which NFAT activation by wild type 3BP2 plays an important role. Consistent with overexpression experiments (42), and studies in mice genetically engineered to express a gain-of-function mutant of 3BP2 (34), our findings also support the importance of NFATc1 in 3BP2-dependent osteoclast signaling. Interestingly, the absence of 3BP2 expression did not affect the acquisition by monocytic cells of a dendritic-like phenotype, as evidenced by the increased expression of dendritic cell markers such as CD11c, CD80 and CD86, observed following stimulation of 3BP2 deficient cells with GM-CSF and IL-4, or upon

further maturation induced by LPS. Early signaling induced by the combination of GM-CSF and IL-4 was also not affected by 3BP2 silencing in RAW264.7 cells. Since myeloid DCs can differentiate upon multiple cytokine signaling pathways (43), a role of 3BP2 in the generation of distinct subsets of DCs cannot be excluded. Recently, a gain-of-function mutant of 3BP2 was involved in increased activity of inflammatory macrophages in a mouse model of Cherubism (34). Thus, it would be interesting to determine whether wild type 3BP2 also participates to the differentiation process of inflammatory DCs and/or macrophages. Nevertheless, our data directly implicates wild type 3BP2 in RANK signaling in the differentiation process of monocytic cells towards osteoclasts. Of note, we recently found that bone marrow macrophages from 3BP2 deficient mice failed to differentiate into osteoclast following RANKL/M-CSF stimulation (Rottapel, R. and Deckert, M., unpublished observations).

The process of osteoclast differentiation from monocytic precursors involves the activation downstream RANK of a large number of signaling molecules and transcription factors (4). It is well established that one crucial event during osteoclastogenesis is the up-regulation of NFATc1, a member of the NFAT family of transcription factors which transcriptional activities are regulated by the Ca2+-dependent phosphatase calcineurin (17). During the early phase of osteoclast precursors differentiation, the initial induction of NFATc1 protein following RANK/RANKL interaction is mediated by a TRAF6-dependent pathway, and leads to the autoamplification of NFATc1, which then regulates the expression of osteoclastogenic genes involved in cell maturation and bone resorption (1,4,17). Differentiation experiments using the RAW264.7 monocyte/macrophage cell line show that absence of 3BP2 in pre-osteoclasts dramatically impaired early and late signaling events typically associated with RANKL stimulation, including actin cytoskeleton remodeling, activation of Src, ERK1/2, JNK, and Akt, and increased expression and activity of c-Fos and NFATc1. As a consequence, the expression of NFATc1 target genes such as TRAP, calcitonin receptor, cathepsin K, and $\alpha V\beta 3$ integrin was severely reduced in the 3BP2-deficient cells following RANK engagement. Interestingly, the expression of the transcription factor PU.1 (data not shown), and of NFATc2 and NFATc3, two other NFAT family members, was not significantly affected by silencing of 3BP2. This suggests that 3BP2 specifically couples RANK signaling to NFATc1 activation, a notion also supported by the rescue experiments with active NFATc1 in 3BP2 knockdown cells. Genetic studies by Aliprantis et al. have established that NFATc1 is downstream of 3BP2 cherubism mutant proteins (34). Together with our observations, this suggests that wild type and mutant 3BP2 proteins function through overlapping signaling pathways. In 3BP2 knockdown macrophages, RANKL stimulation failed to induce c-Fos mRNA expression and AP-1 activity, suggesting that 3BP2 participate to the initial TRAF6-dependent induction of NFATc1 messenger expression, rather than to the NFATc1-dependent amplification of NFATc1 induction. TRAF6 and Src physically interact during osteoclast activation (5), and Src phosphorylation was impaired downstream of RANK in the absence of 3BP2. Conversely, the expression of a gain-of-function ''cherubism' mutant of 3BP2 (3BP2 R415P) in RAW264.7 cells led to enhanced osteoclast formation and increased Src phosphorylation, underlining the importance of Src for 3BP2 signaling in osteoclasts. Importantly, we found that c-Src physically interacted with 3BP2 in monocytic cells. Therefore, it should be interesting to examine whether 3BP2 interacts with TRAF6, directly or indirectly through Src or additional proteins.

Constitutively active Src only partially rescued the impaired osteoclast differentiation observed in 3BP2 knockdown cells. Although osteoclasts rescued by overexpression of active Src differentiated into large multinucleated cells expressing high levels of TRAP, their morphology and size suggested that they are not fully mature. Thus, another question is how does 3BP2 couple RANK to the activation of NFATc1 besides Src? Interestingly, various binding partners of 3BP2, identified in other leukocytes (29, 30), have been involved in bone development, including Abl (44), Src (10), PLCγ (45), Cbl (46), and Vav3 (8). Moreover, the costimulatory signals provided by the ITAM-containing adaptor proteins DAP12 and FcRγ have been shown to play critical roles during osteoclast differentiation through Syk (6,7). Osteoclasts from DAP12 x FcRγ-null mice exhibit defective NFATc1 expression as a consequence of impaired PLCγ-dependent

calcium signaling (6). Of note, B cells from 3BP2-/- mice show impaired PLCγ phosphorylation, calcium mobilization and NFAT activation (24). Our observations that in myeloid cells 3BP2 forms a signaling complex with c-Src, Syk, Vav and Cbl provide a mechanistic explanation on how 3BP2 might participate to RANK-mediated osteoclast formation. Also, overexpression of active Src in control and 3BP2 knockdown cells induced the phosphorylation of Syk and ERK1/2, two events critically involved in several aspects of osteoclast function (2). Thus, by regulating Src activity, 3BP2 might impact on other osteoclast signaling pathways. Interestingly, Src-induced phosphorylation of Cbl downstream αvβ3 integrin promotes the recruitment and activation of PI3K in a signaling complex composed of Pyk2-Src-Cbl essential for osteoclast bone-resorbing activity (51). The interaction of 3BP2 with Cbl in RAW264.7 cells therefore suggests that phosphorylation of Cbl by c-Src represent another possible event of 3BP2 signaling in osteoclast. Src-/- mice are osteopetrotic because of defective formation of ruffled borders and subsequent bone resorption, but not because of impaired osteoclast differentiation (47), suggesting the existence of compensatory mechanisms by other Src family members in mice. Whereas a negative regulatory function of Lyn in osteoclastogenesis was reported (48), the expression of Fyn, another Src family member, increased during osteoclast differentiation (12). Our observations that 3BP2 is a partner and substrate of Fyn (21,23) therefore suggest that Fyn may participate to 3BP2 signaling in osteoclast apart Src.

In the absence of 3BP2, osteoclast precursors failed to reorganize their actin cytoskeleton, a process that has been found actively regulated by Src and Syk kinases, as well as Rho GTPases and GEFs proteins, such as Vav family members (1,8,49). The observed interactions of 3BP2 with Src, Syk and Vav in myeloid cells again provide some mechanistic insights on how 3BP2 might participate to cytoskeleton reorganization in forming osteoclasts. In genetically engineered mice, a gain-of-function mutant of 3BP2 promoted increased phosphorylation of Syk (32). Interestingly, we observed that a 3BP2 cherubism mutant expressed in macrophages strongly increased Src phosphorylation, compared to wild type 3BP2. It is therefore likely that 3BP2 regulates the localization and/or the activity of some of these proteins through its different protein binding domains. Further experiments using co-cultured primary bone marrow macrophages and osteoblasts, which bring adhesion receptor ligands, should clarify the mechanism by which endogenous 3BP2 regulates osteoclast actin cytoskeleton remodeling. Interestingly, Src and Syk kinases signaling pathways are involved downstream multiple receptors necessary to osteoclast differentiation, ruffled borders formation and bone resorption, including RANK, costimulatory Immunoreceptors coupled to ITAM-containing adapter proteins DAP12/FcRγ, αvβ3 integrin, and c-fms (11,13,50). Thus, 3BP2 may integrate osteoclastogenic signals through its interactions with Src, Syk, Vav and Cbl proteins.

In conclusion, our study provides evidence that wild type 3BP2 is a crucial regulator of RANK-mediated macrophage differentiation into osteoclast. Further studies aimed at elucidating the molecular mechanisms by which wild type and pathological 3BP2 proteins regulate leukocyte activation and differentiation should help a better understanding of inflammatory and bone diseases.

REFERENCES

1. Boyle, W. J., Simonet, W. S., and Lacey, D. L. (2003) *Nature* **423**, 337-342
2. Walsh, M. C., Kim, N., Kadono, Y., Rho, J., Lee, S. Y., Lorenzo, J., and Choi, Y. (2006) *Annu Rev Immunol* **24**, 33-63
3. Kong, Y. Y., Yoshida, H., Sarosi, I., Tan, H. L., Timms, E., Capparelli, C., Morony, S., Oliveira-dos-Santos, A. J., Van, G., Itie, A., Khoo, W., Wakeham, A., Dunstan, C. R., Lacey, D. L., Mak, T. W., Boyle, W. J., and Penninger, J. M. (1999) *Nature* **397**, 315-323
4. Teitelbaum, S. L., and Ross, F. P. (2003) *Nat Rev Genet* **4**, 638-649
5. Wong, B. R., Besser, D., Kim, N., Arron, J. R., Vologodskaia, M., Hanafusa, H., and Choi, Y. (1999) *Mol Cell* **4**, 1041-1049
6. Koga, T., Inui, M., Inoue, K., Kim, S., Suematsu, A., Kobayashi, E., Iwata, T., Ohnishi, H.,

Matozaki, T., Kodama, T., Taniguchi, T., Takayanagi, H., and Takai, T. (2004) *Nature* **428**, 758-763
7. Mocsai, A., Humphrey, M. B., Van Ziffle, J. A., Hu, Y., Burghardt, A., Spusta, S. C., Majumdar, S., Lanier, L. L., Lowell, C. A., and Nakamura, M. C. (2004) *Proc Natl Acad Sci U S A* **101**, 6158-6163
8. Faccio, R., Teitelbaum, S. L., Fujikawa, K., Chappel, J., Zallone, A., Tybulewicz, V. L., Ross, F. P., and Swat, W. (2005) *Nat Med* **11**, 284-290
9. Munugalavadla, V., Vemula, S., Sims, E. C., Krishnan, S., Chen, S., Yan, J., Li, H., Niziolek, P. J., Takemoto, C., Robling, A. G., Yang, F. C., and Kapur, R. (2008) *Mol Cell Biol* **28**, 7182-7198
10. Soriano, P., Montgomery, C., Geske, R., and Bradley, A. (1991) *Cell* **64**, 693-702
11. Zou, W., Kitaura, H., Reeve, J., Long, F., Tybulewicz, V. L., Shattil, S. J., Ginsberg, M. H., Ross, F. P., and Teitelbaum, S. L. (2007) *J Cell Biol* **176**, 877-888
12. Shinohara, M., Koga, T., Okamoto, K., Sakaguchi, S., Arai, K., Yasuda, H., Takai, T., Kodama, T., Morio, T., Geha, R. S., Kitamura, D., Kurosaki, T., Ellmeier, W., and Takayanagi, H. (2008) *Cell* **132**, 794-806
13. Zou, W., Reeve, J. L., Liu, Y., Teitelbaum, S. L., and Ross, F. P. (2008) *Mol Cell* **31**, 422-431
14. Takayanagi, H., Kim, S., Koga, T., Nishina, H., Isshiki, M., Yoshida, H., Saiura, A., Isobe, M., Yokochi, T., Inoue, J., Wagner, E. F., Mak, T. W., Kodama, T., and Taniguchi, T. (2002) *Dev Cell* **3**, 889-901
15. Asagiri, M., Sato, K., Usami, T., Ochi, S., Nishina, H., Yoshida, H., Morita, I., Wagner, E. F., Mak, T. W., Serfling, E., and Takayanagi, H. (2005) *J Exp Med* **202**, 1261-1269
16. Hogan, P. G., Chen, L., Nardone, J., and Rao, A. (2003) *Genes Dev* **17**, 2205-2232
17. Takayanagi, H. (2007) *Ann N Y Acad Sci* **1116**, 227-237
18. Hirotani, H., Tuohy, N. A., Woo, J. T., Stern, P. H., and Clipstone, N. A. (2004) *J Biol Chem* **279**, 13984-13992
19. Samelson, L. E. (2002) *Annu Rev Immunol* **20**, 371-394
20. Kurosaki, T. (2002) *Curr Opin Immunol* **14**, 341-347
21. Deckert, M., Tartare-Deckert, S., Hernandez, J., Rottapel, R., and Altman, A. (1998) *Immunity* **9**, 595-605
22. Le Bras, S., Moon, C., Foucault, I., Breittmayer, J. P., and Deckert, M. (2007) *FEBS Lett* **581**, 967-974
23. Foucault, I., Le Bras, S., Charvet, C., Moon, C., Altman, A., and Deckert, M. (2005) *Blood* **105**, 1106-1113
24. de la Fuente, M. A., Kumar, L., Lu, B., and Geha, R. S. (2006) *Mol Cell Biol* **26**, 5214-5225
25. Chen, G., Dimitriou, I. D., La Rose, J., Ilangumaran, S., Yeh, W. C., Doody, G., Turner, M., Gommerman, J., and Rottapel, R. (2007) *Mol Cell Biol* **27**, 3109-3122
26. Shukla, U., Hatani, T., Nakashima, K., Ogi, K., and Sada, K. (2009) *J Biol Chem*
27. Jevremovic, D., Billadeau, D. D., Schoon, R. A., Dick, C. J., and Leibson, P. J. (2001) *J Immunol* **166**, 7219-7228.
28. Sada, K., Miah, S. M., Maeno, K., Kyo, S., Qu, X., and Yamamura, H. (2002) *Blood* **100**, 2138-2144.
29. Deckert, M., and Rottapel, R. (2006) *Adv Exp Med Biol* **584**, 107-114
30. Hatani, T., and Sada, K. (2008) *Curr Med Chem* **15**, 549-554
31. Ueki, Y., Tiziani, V., Santanna, C., Fukai, N., Maulik, C., Garfinkle, J., Ninomiya, C., doAmaral, C., Peters, H., Habal, M., Rhee-Morris, L., Doss, J. B., Kreiborg, S., Olsen, B. R., and Reichenberger, E. (2001) *Nat Genet* **28**, 125-126.
32. Ueki, Y., Lin, C. Y., Senoo, M., Ebihara, T., Agata, N., Onji, M., Saheki, Y., Kawai, T., Mukherjee, P. M., Reichenberger, E., and Olsen, B. R. (2007) *Cell* **128**, 71-83
33. Southgate, J., Sarma, U., Townend, J. V., Barron, J., and Flanagan, A. M. (1998) *J Clin Pathol* **51**, 831-837
34. Aliprantis, A. O., Ueki, Y., Sulyanto, R., Park, A., Sigrist, K. S., Sharma, S. M., Ostrowski, M. C., Olsen, B. R., and Glimcher, L. H. (2008) *J Clin Invest* **118**, 3775-3789
35. Bailet, O., Fenouille, N., Abbe, P., Robert, G., Rocchi, S., Gonthier, N., Denoyelle, C., Ticchioni, M., Ortonne, J. P., Ballotti, R., Deckert, M., and Tartare-Deckert, S. (2009) *Cancer*

Res **69**, 2748-2756
36. Sallusto, F., and Lanzavecchia, A. (1994) *J Exp Med* **179**, 1109-1118
37. Faccio, R., Novack, D. V., Zallone, A., Ross, F. P., and Teitelbaum, S. L. (2003) *J Cell Biol* **162**, 499-509
38. Saltel, F., Chabadel, A., Bonnelye, E., and Jurdic, P. (2008) *Eur J Cell Biol* 87 , 459-468
39. Thomas, S. M., and Brugge, J. S. (1997) *Annu Rev Cell Dev Biol* **13**, 513-609
40. Monticelli, S., and Rao, A. (2002) *Eur J Immunol* **32**, 2971-2978
41. Aramburu, J., Yaffe, M. B., Lopez-Rodriguez, C., Cantley, L. C., Hogan, P. G., and Rao, A. (1999) *Science* **285**, 2129-2133
42. Lietman, S. A., Yin, L., and Levine, M. A. (2008) *Biochem Biophys Res Commun* **371**, 644-648
43. Shortman, K., and Naik, S. H. (2007) *Nat Rev Immunol.* **7**, 19-30
44. Li, B., Boast, S., de los Santos, K., Schieren, I., Quiroz, M., Teitelbaum, S. L., Tondravi, M. M., and Goff, S. P. (2000) *Nat Genet* **24**, 304-308
45. Mao, D., Epple, H., Uthgenannt, B., Novack, D. V., and Faccio, R. (2006) *J Clin Invest* **116**, 2869-2879
46. Tanaka, S., Amling, M., Neff, L., Peyman, A., Uhlmann, E., Levy, J. B., and Baron, R. (1996) *Nature* **383**, 528-531
47. Boyce, B. F., Yoneda, T., Lowe, C., Soriano, P., and Mundy, G. R. (1992) *J Clin Invest* **90**, 1622-1627
48. Kim, H. J., Zhang, K., Zhang, L., Ross, F. P., Teitelbaum, S. L., and Faccio, R. (2009) *Proc Natl Acad Sci U S A* **106**, 2325-2330
49. Ory, S., Brazier, H., Pawlak, G., and Blangy, A. (2008) *Eur J Cell Biol* **87**, 469-477
50. Insogna, K. L., Sahni, M., Grey, A. B., Tanaka, S., Horne, W. C., Neff, L., Mitnick, M., Levy, J. B., and Baron, R. (1997) *J Clin Invest* **100**, 2476-2485
51. Miyazaki, T., Sanjay, A., Neff, L., Tanaka, S., Horne, W. C., and Baron, R. (2004) *J Biol Chem* **279**, 17660-17666

FOOTNOTES

This work was supported by INSERM, ANR-MRAR, and Association pour la Recherche sur le Cancer. AG was a recipient of a fellowship from the Ligue Nationale Contre le Cancer. VP is supported by the Fondation pour la Recherche Médicale. M. Deckert was a recipient of a Contrat d'Interface Clinique with the Department of Clinical Hematology, CHU de Nice (France). We thank A. Rao and J. Brugge for VIVIT and CA-NFAT, and Src Y527F expression plasmids, respectively, N. Taylor for the LZRS-IRES-GFP retroviral vector, H. Takayanagi for TRAP promoter-luciferase construct. We also thank N. Gonthier and A. Mallavialle for Q-PCR analysis, and A. Wakkach for helpful discussions.

Abbreviations used: 3BP2, c-Abl SH3 domain-binding protein-2; RANK, receptor activator of NFκB; RANKL, RANK, receptor activator of NFκB ligand; GM-CSF; granulocyte macrophage-colony stimulating factor; NFκB, nuclear factor κB; NFAT, nuclear factor of activated T cells; TRAP, tartrate resistant alkaline phosphatase; PTK, protein-tyrosine kinase; SH2, Src homology 2; PLC, phospholipase C; IKK, IκB kinase; ITAM, immunoreceptor tyrosine-based activation motif; sh, short hairpin.

FIGURE LEGENDS

Fig. 1. Expression of 3BP2 is required for RANKL-stimulated osteoclast formation in RAW264.7 cells. (A) Generation of stable 3BP2 knockdown in RAW264.7 cells. Three short hairpin RNA (shRNA) expressing plasmids targeting position 381-401 (381), 488-508 (488) and 507-527 (507) of mouse 3BP2 RNA, and a control LacZ shRNA construct were stably transfected into RAW264.7 cells. After selection, stable suppression of 3BP2 expression was evaluated by

immunoblotting using antibodies against 3BP2 and ERK2 as loading control. **(B)** 3BP2 knockdown cells (sh3BP2 (381), (488) and (507), and shLacZ control cells were cultured for 4 days with sRANKL (40 ng/ml), and stained for TRAP activity. Morphology (top panels) and multinucleated TRAP-positive cells (bottom panels) were assessed and scored by microscopy. To determine complexity and size difference, TRAP+ multinucleated cell number and nuclei per osteoclast were counted. Data are expressed as the mean ± SD of 3 equivalent wells and are representative of 3 independent experiments. **, $p < 0.01$ *versus* shLacZ; ***, $p < 0.001$ *versus shLacZ.* Scale bars = 50 μM. **(C)** shLacZ and sh3BP2 (381) cells were transfected with a TRAP luciferase reporter construct. 8 hours after transfection, cells were stimulated or not with RANKL (40 ng/ml). Normalized luciferase activity was determined 24 hours after stimulation and expressed as the fold increase relative to basal activities measured in control vector-transfected cells. Results are the mean ± SD of triplicate determinations. ***, $p < 0.001$ *versus* shLacZ. **(D)** shLacZ and sh3BP2 cells were stimulated or not with RANKL (40 ng/ml) for the indicated times. The expression of TRAP, calcitonin receptor and cathepsin K mRNA was determined by real-time quantitative PCR. Data are expressed as the mean ± S.D of triplicate determinations and are representative of three independent experiments.

Fig. 2. Effects of 3BP2 suppression on differentiation of RAW264.7 cells into dendritic cells. (A) shLacZ and sh3BP2 cells were cultured with GM-CSF (100 ng/ml) and IL-4 (10 ng/ml) for 6 days and then activated for 2 additional days with LPS (10 ng/ml). Dendritic-like cell morphology was examined by light microscopy. A representative field of each culture condition is shown. Scale bars = 50 μM. **(B)** Cells were cultured as described above and stained with FITC-, or phycoerythrin-conjugated antibodies against CD11c, CD80 or CD86, followed by flow cytometry analysis. Dotted line histograms represent cells stained with isotype-matched control antibodies. The data shown are representative of three independent experiments. **(C)** Cell lines were cultured in the absence of serum for 12 hours before stimulation with GM-CSF (100 ng/ml) and IL-4 (10 ng/ml) for 0, 15, 30 and 60 minutes. Cell lysates were subjected to immunoblot analysis with antibodies against phospho-p38, -IKKα/β, -AKT, and - ERK1/2. The same membrane was stripped and reprobed with anti-ERK2.

Fig. 3. Effects of 3BP2 suppression on RANKL-induced organization of the osteoclast actin cytoskeleton. (A) shLacZ and sh3BP2 cells were seeded onto sterile chamber slide treated with sRANKL (40 ng/ml) for the indicated times. After washing, cells were fixed, permeabilized and incubated with 100 ng/ml of TexasRed phalloidin for 30 min. Polymerized actin was visualized using fluorescence microscope. The data shown are representative of three independent experiments. Scale bars = 50 μM. **(B)** Cells were treated with sRANKL (40 ng/ml) for the indicated times. Following cell fixation and labeling with AlexaFluor 488-conjugated phalloidin (100 ng/ml), actin polymerization was quantified by flow cytometry. Data are expressed as mean channel fluorescence intensity for each sample. Results represent the mean ± SD of three independent determinations. **(C)** shLacZ and sh3BP2 cells were stimulated or not with RANKL (40 ng/ml) for the indicated times. The expression of integrin αv and β3 subunits, and CD44 mRNA was determined by real-time quantitative PCR. Data are expressed as the mean ± S.D of triplicate determinations and are representative of three independent experiments. **(D)** Cells were cultured without serum for 12 hours before stimulation with sRANKL (100 ng/ml) for 0, 10, and 60 minutes. Cell lysates were subjected to immunoblot analysis with antibodies against phospho-ERM, and phospho-FAK. The same membrane was stripped and reprobed with anti-ERK2.

Fig. 4. 3BP2 knockdown affects multiple signaling pathways in response to RANKL. (A) shLacZ and sh3BP2 cells were cultured in the absence of serum for 12 hours before stimulation with sRANKL (100 ng/ml) for 0, 10, and 60 minutes. Cell lysates were separated by SDS-PAGE and transferred to nitrocellulose membrane that were subjected to immunoblot analysis with antibodies against phospho-Src, -ERK1/2, -JNK, -p38, -AKT, -IKKα/β, and –MEK1/2. After stripping, the membrane was reprobed with anti-3BP2, Src, AKT, MEK2 and ERK2. **(B)** shLacZ (black bars) and sh3BP2 (white bars) cells were transfected with AP-1 or NFAT luciferase reporter constructs. 8 hours after transfection, cells were stimulated or not with sRANKL (100 ng/ml). Normalized luciferase activity

was determined 24 hours after stimulation and expressed as the fold increase relative to basal activities measured in control vector-transfected cells. Results are the mean ± SD of triplicate determinations. ***, $p < 0.001$ *versus* shLacZ. **(C)** Cells were stimulated with sRANKL for the indicated times. The expression of c-Fos, Src, NFATc1, NFATc2, and NFATc3 mRNA was determined by real-time quantitative PCR. Data are expressed as the mean ± S.D of triplicate determinations and are representative of three independent experiments. **(D)** Lysates from cells stimulated as indicated above were subjected to immunoblot analysis with antibodies against NFATc1, Src, and 3BP2. Protein loading was controlled by reprobing the membrane with anti-ERK2.

Fig. 5. Ectopic expression of active Src Y527 protein partially rescues RANKL-induced osteoclast formation in the absence of 3BP2 in RAW264.7 cells. **(A)** 3BP2 interacts with c-Src, Syk, Vav1, and Cbl in resting RAW264.7 cells. Cells were lysed at $1x10^8$ cells/ml in ice-cold lysis buffer (1% Triton in 150 mM NaCl, 50 mM Tris-HCl [pH 7.5], 0.1% SDS, 0.1% sodium deoxycholate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF) for 30 min on ice. Cleared lysates from $1x10^7$ cells were incubated with nonrelevant goat antibodies (NR) or sheep anti-3BP2 antibodies for 3h at 4°C followed by incubation with protein G-Sepharose beads for 1 h. After three washes, immunoprecipitates were immunoblotted using antibodies against 3BP2, c-Src, Syk, Vav1, and Cbl. WCL: whole cell lysate. **(B)** shLacZ and sh3BP2 cells were transfected with pLNCX srcY527F retroviral construct or control vector, together with EGFP expression vector. After 48 hours, the GFP-positive cells were purified on a FACSAria cell sorter, and subjected to immunoblotting analysis using antibodies against 3BP2, phospho-Src, phospho-Syk, and phospho-ERK1/2. Protein loading was controlled with anti-ERK2 immunoblot. Cells purified as indicated above were cultured for 4 days with sRANKL (40 ng/ml), and stained for TRAP activity. Morphology **(C)** and multinucleated TRAP-positive cells **(D)** were assessed and scored by microscopy. Scale bars = 50 μM. TRAP+ multinucleated cell number and nuclei per osteoclast were counted. Data are expressed as the mean ± SD of 3 equivalent wells and are representative of 3 independent experiments. ***, $p < 0.001$ *versus* shLacZ.

Fig. 6. Complementation of osteoclast formation by ectopic expression of constitutively active NFATc1 in 3BP2 knockdown cells. shLacZ and sh3BP2 cells were transfected with control vector or pRV-HA-caNFAT2-IRES-GFP vector encoding HA-tagged constitutively active caNFATc1 (caNFAT). After 48 hours, the GFP-positive cells were purified on a FACSAria cell sorter. **(A)** Purified cells were subjected to immunoblotting analysis using antibodies against HA tag. Protein loading was controlled with anti-ERK2 immunoblot. Cells purified as indicated above were then cultured for 4 days in the presence of sRANKL (40 ng/ml), and stained for TRAP activity. Morphology **(B)** and multinucleated TRAP-positive cells **(C)** were scored by microscopy. Cells with more than three nuclei were counted multinucleated cells and nuclei per osteoclast were counted. Data are expressed as the mean ± SD of 3 independent determinations. **, $p < 0.01$ *versus* shLacZ; ***, $p < 0.001$ *versus* shLacZ.

Fig. 7. Effects of the expression of a 3BP2 cherubism mutant on Src activation and osteoclast differentiation in RAW264.7 cells. RAW264.7 cells were transfected with control vector, LZRS-V5-3BP2-IRES-GFP, or LZRS-V5-3BP2 R415P. **(A)** GFP-positive cells were purified on a FACSAria cell sorter. **(B)** Sorted cells were stimulated for 5 days with sRANKL (40 ng/ml), and stained for TRAP activity. Morphology and multinucleated TRAP-positive cells were scored by microscopy as described above. Scale bars = 50 μM. Data are expressed as the mean ± SD of 3 independent determinations. **, $p < 0.01$ *versus* shLacZ; ***, $p < 0.001$ *versus* shLacZ. **(C)** Purified cells were subjected to immunoblotting analysis using antibodies against V5 tag, 3BP2, phospho-Src Y416. Protein loading was controlled with anti-Src and ERK2.

Figure 1

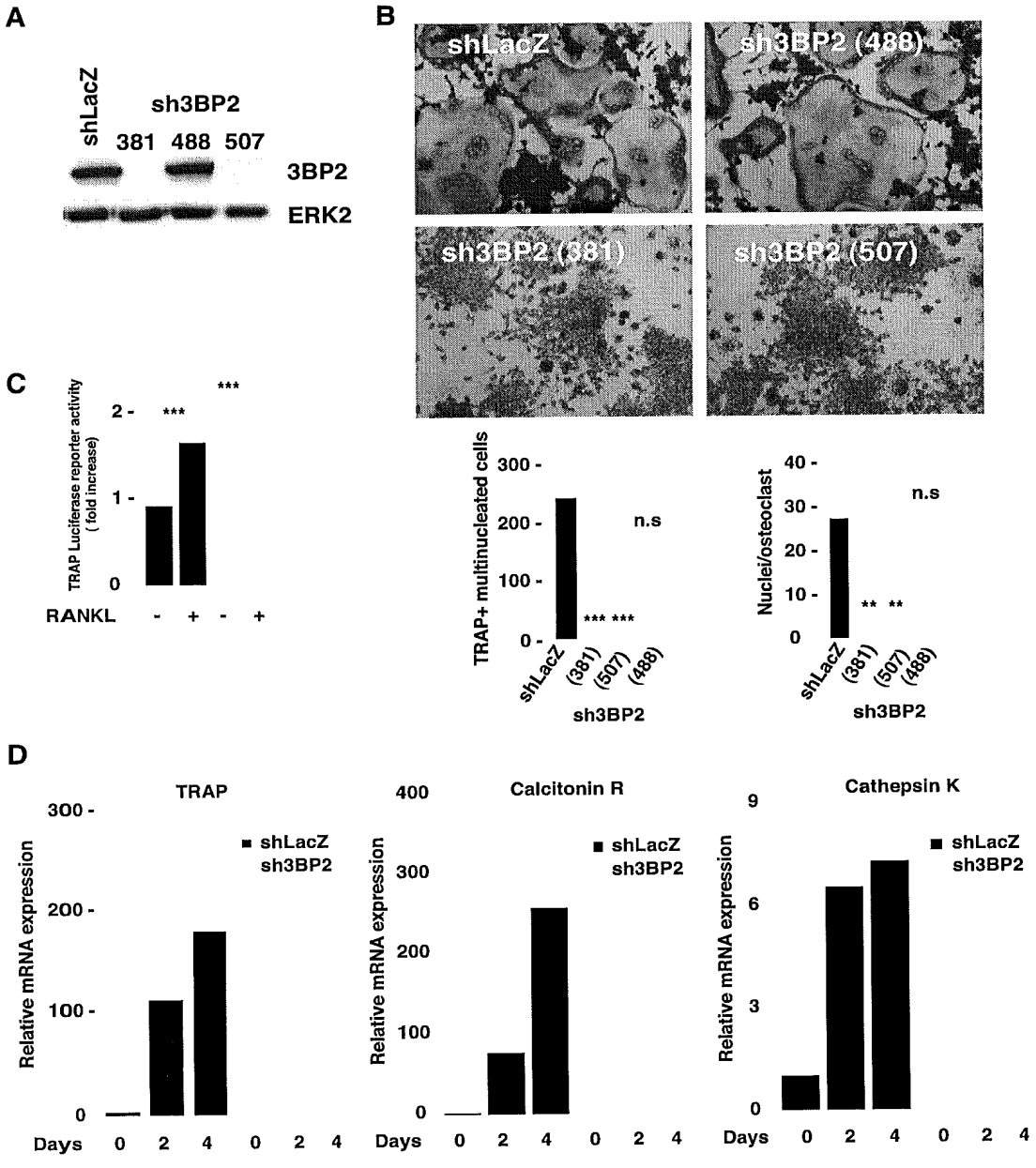


Figure 2

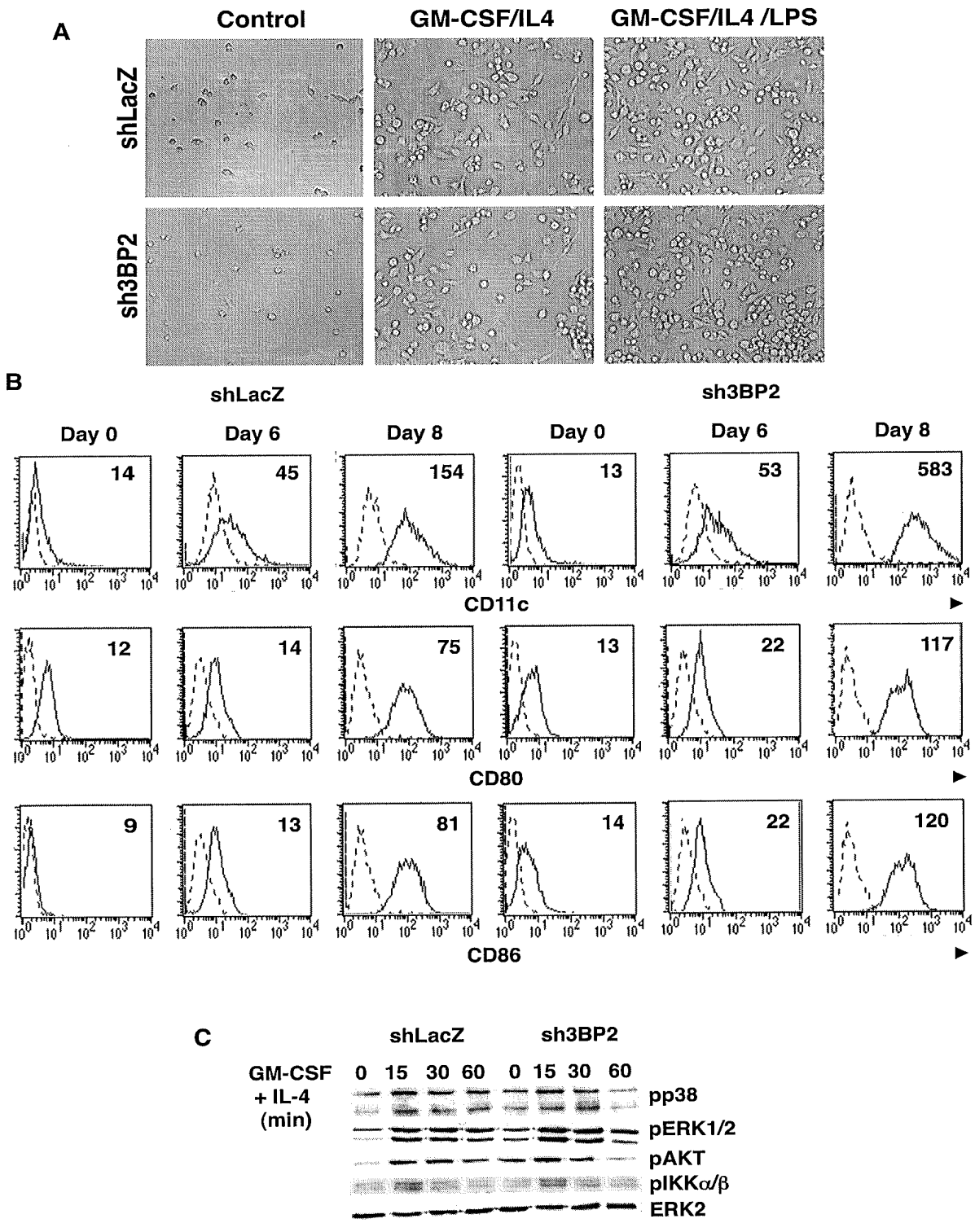


Figure 3

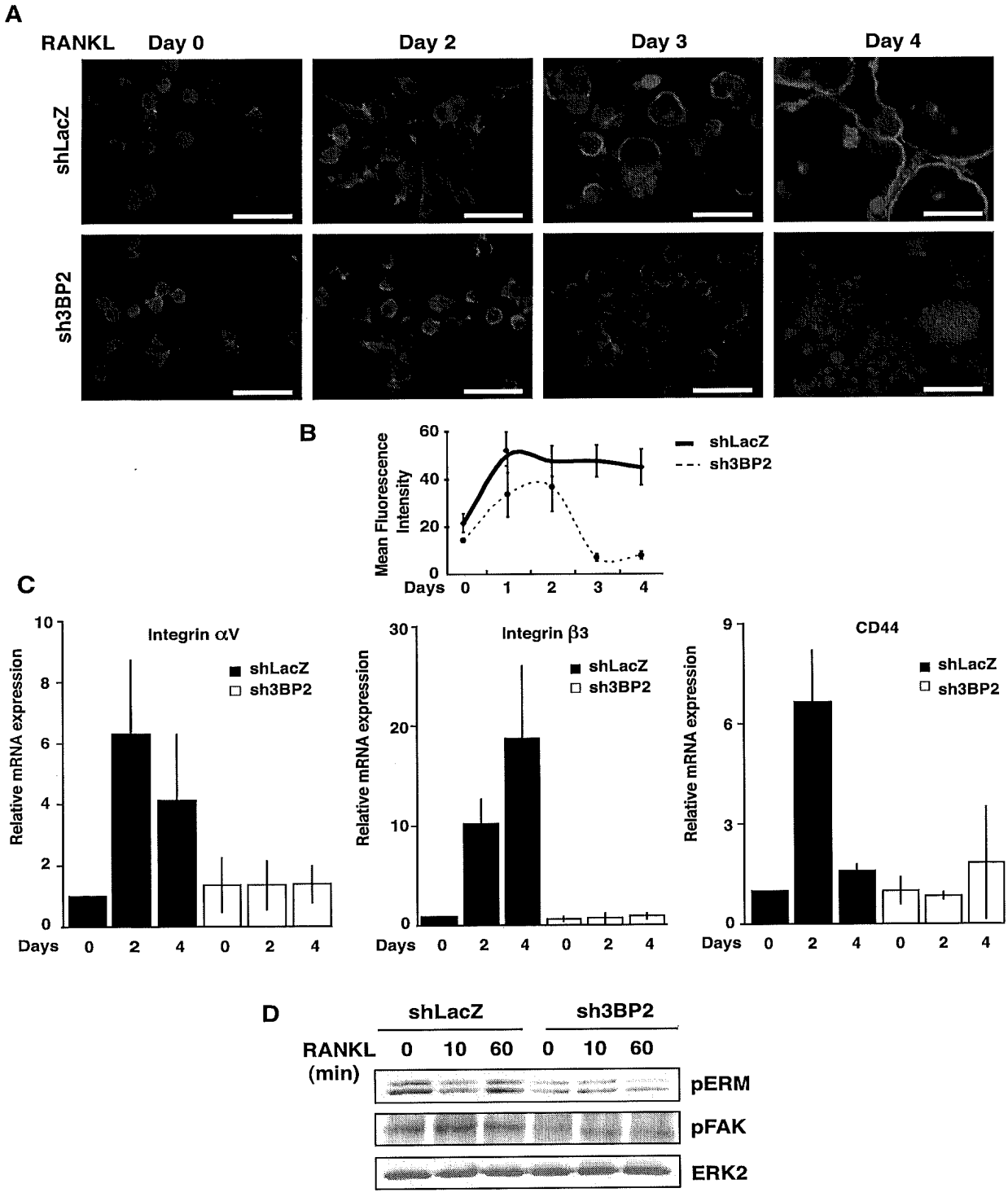
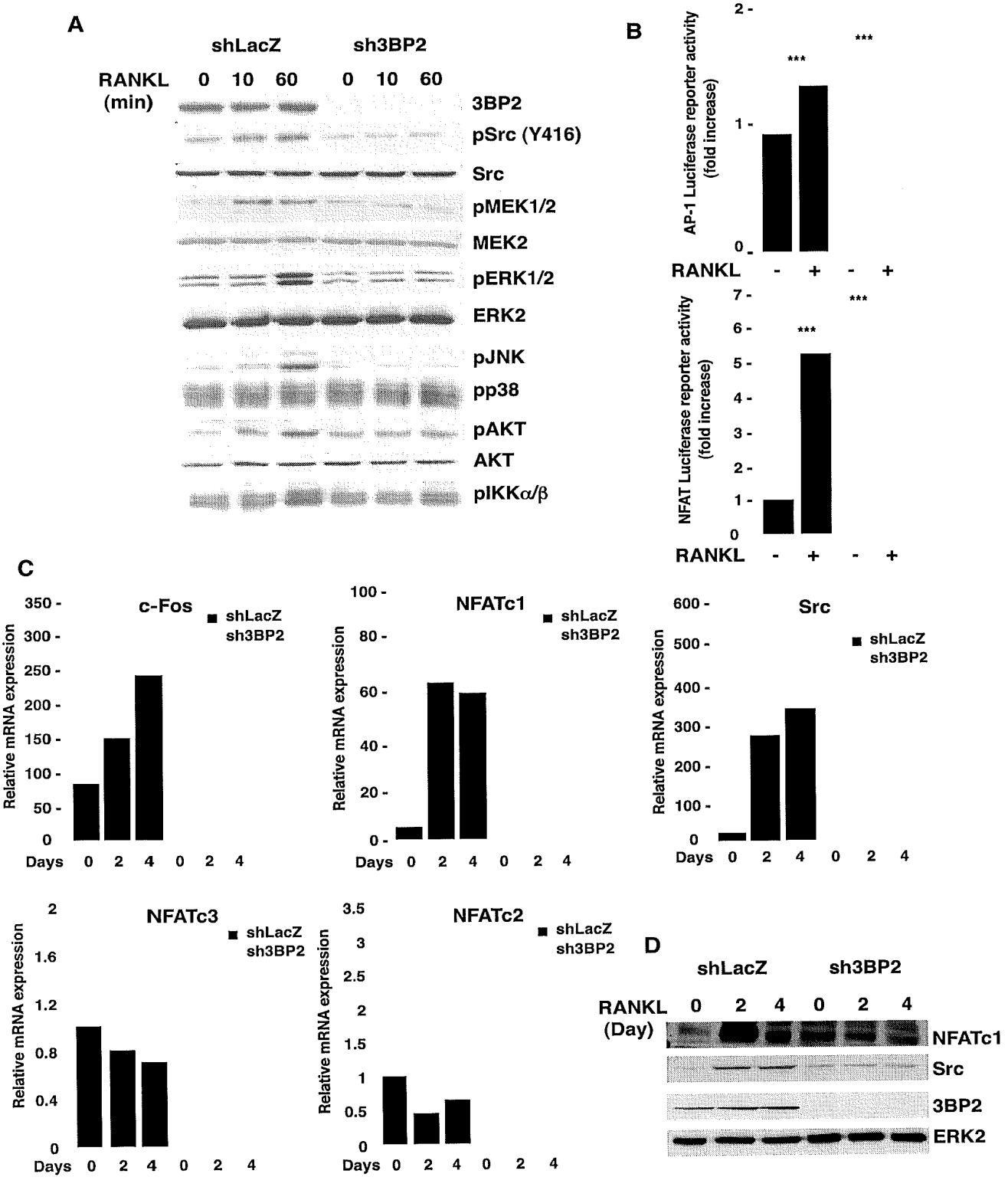

A
RANKL
Day 0
Day 2
Day 3
Day 4
shLacZ
sh3BP2
B
Mean Fluorescence Intensity
60
40
20
0
Days 0 1 2 3 4
shLacZ
sh3BP2
C
Integrin αV
Relative mRNA expression
10
8
6
4
2
0
shLacZ
sh3BP2
Days 0 2 4 0 2 4
Integrin β3
30
20
10
0
Days 0 2 4 0 2 4
CD44
9
6
3
0
Days 0 2 4 0 2 4
D
shLacZ
sh3BP2
RANKL (min) 0 10 60 0 10 60
pERM
pFAK
ERK2

Figure 4



New Figure 5

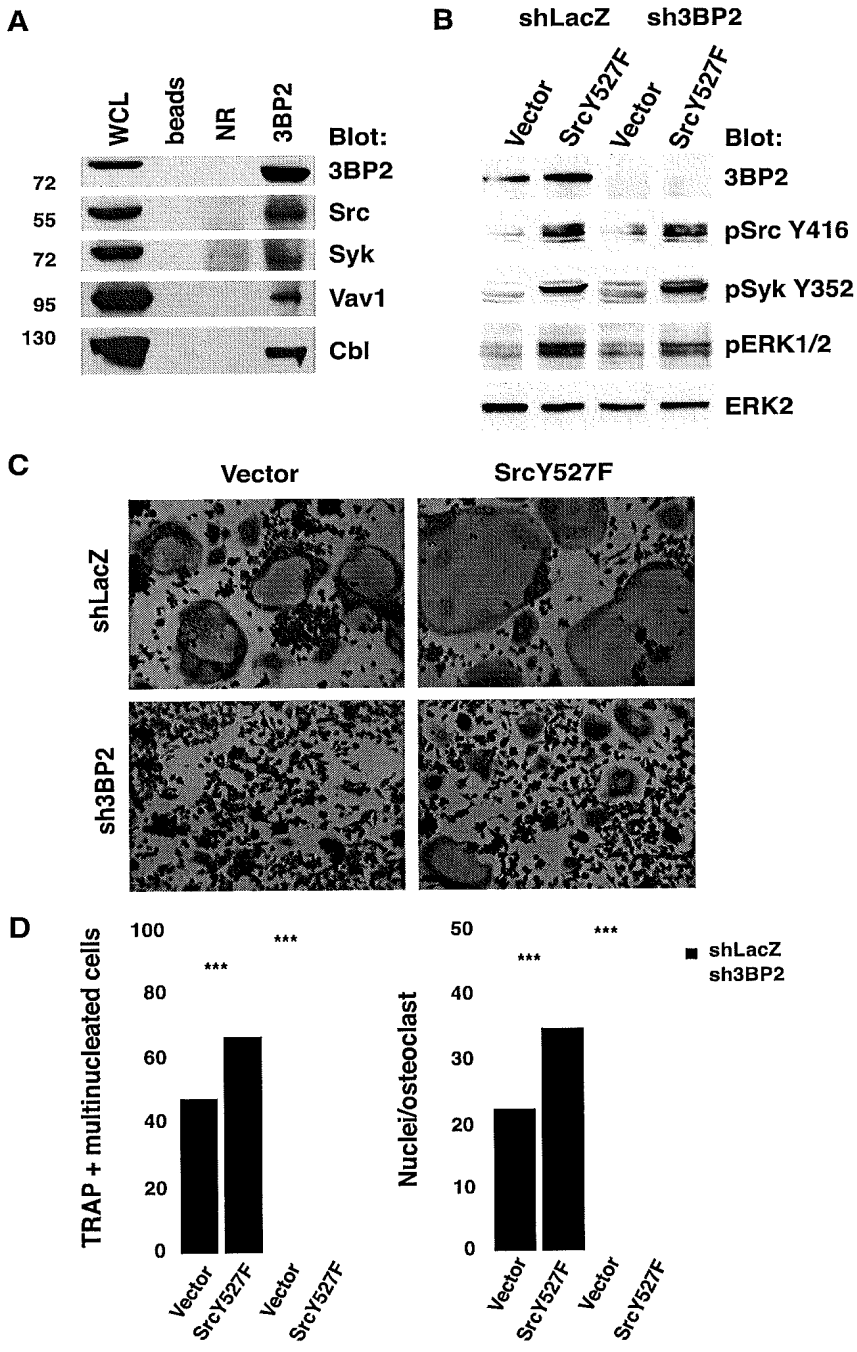
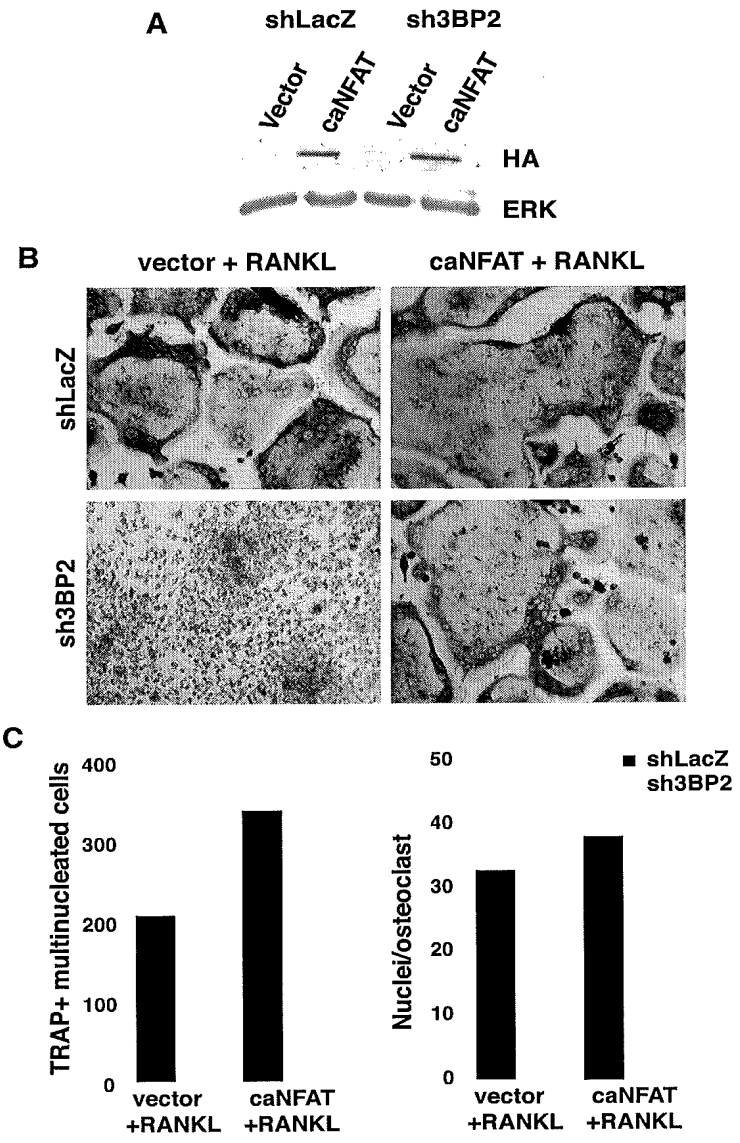


Figure 6



New Figure 7